Preincubation of *Rhizobium leguminosarum* bv. *phaseoli* with jasmonate and genistein signal molecules increases bean (*Phaseolus vulgaris* L.) nodulation, nitrogen fixation and biomass production

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**Abstract**

Jasmonates, Jasmonic acid (JA) and Methyl Jasmonate (MeJA), are naturally occurring hormones in plants. Their role in induced disease resistance and other biotic and abiotic stress responses of the plants is well characterized. They are also rhizosecreted from roots into the rhizosphere; however their role in the rhizosphere and their effect on microbial populations has been largely uninvestigated. We have previously shown that jasmonates can induce *nod* genes and the production of lipo-chitooligosaccharides (LCOs) from rhizobia, and are thus potentially important signalling molecules in rhizobia-legume symbioses. We have already shown that jasmonate preincubated rhizobia can enhance nodulation and nitrogen fixation of soybean plants. The objective of the reported work was to study the effect of *Rhizobium leguminosarum* bv. *phaseoli* pre-induced with genistein, MeJA or both on nodulation, nitrogen fixation and early growth of common bean (*Phaseolus vulgaris*) plants. Both genistein and MeJA enhanced nodule number, nitrogen content per plant, and plant dry matter accumulation. Genistein and MeJA, when applied to cultures together, showed synergistic effects on all studied variables. These results demonstrate that rhizobial cells treated with either inducer improve bean nodulation, nitrogen fixation and growth, and that those treated with both are most effective. Enhanced nitrogen fixation led to more nitrogen translocation from root nodules to shoots, thus increasing leaf nitrogen content of plants. Bean plants inoculated with preinduced rhizobia had elevated photosynthetic rates, contributing to increased plant dry matter accumulation. The results of this study indicate that MeJA alone or in combination with genistein can be used to promote bean nodulation, nitrogen fixation and early plant growth.

**Key words:** Genistein, Jasmonates, Nitrogen fixation, Nodulation, *Phaseolus vulgaris*, *Rhizobium leguminosarum* bv. *phaseoli*

**Introduction**

Nitrogen fertilizer has now become a major environmental problem and a health hazard (Addiscott, 1996, Zahran, 1999); symbiotic N2 fixation is a sustainable alternative. Common bean is a nitrogen fixing legume and one of the world’s most important sources of dietary protein (Hantngston et al., 1986). In spite of its ability to fix nitrogen, the addition of nitrogen fertilizers is usually recommended for bean production (Piha and Muns, 1987). This is because common bean appears to be inferior to other grain legumes in nodulation and N2 fixation (Richardson et al. 1988, Bandyopadhyay et al. 1996, and Graham, 1984). However, it is generally thought that nitrogen fixation in bean is not genetically inferior (Franco and Munns, 1981), rather that host variety and *Rhizobium* strain incompatibilities contribute to the frequently unsatisfactory response of bean to inoculation under field condition (Graham, 1981).
Like other legume crops, there have been many investigations of ways to improve symbiotic nitrogen fixation by bean, leading to yield improvement. However, the bean symbiotic N₂ fixation system seems unable to provide sufficient nitrogen, and *Rhizobium* inoculants are seldom used to reduce bean dependence on combined nitrogen, since the response of bean to inoculation has proved too variable (Graham, 1981). The use of higher inoculation rates, modified inoculates carriers, and strains selected for competitiveness in nodulation have all failed to overcome this problem (Montealegro *et al.* 1995). There have been a few reports of increased seed yield following rhizobial inoculation (e.g., Park and Buttery, 1989), however, many other reports indicate that inoculation with commercial strains, compared to an uninoculated control, fail to increase the seed yield (Weiser *et al.* 1985 Richardson *et al.* 1988, Chavera and Graham 1992). An increase in the rhizospheric concentration of *nod* gene inducers through exogenous application has been reported for some legumes, but not bean (Bandyopadhyay *et al.* 1996 and Pan *et al.* 1998).

Among the rhizospheric factors potentially influencing nitrogen fixation by symbiotic systems, plant molecular signals involved in plant-rhizobia interactions are promising. It is reported that the presence of appropriate flavonoids in root exudates is an important contributing factor to the nodule formation (Richardson *et al.* 1988). The nodulation status (nodule number and nodule weight) of pea is reported to be improved by addition of the flavonoid naringenin (Bandyopadhyay *et al.* 1996). Several studies have documented the use of flavonoid inducer molecules as a tool in enhancing nodulation and nitrogen fixation (Davis and Johnston, 1990, Bandyopadhyay *et al.* 1996, Pan and Smith, 1998). Isoflavonoid application to the rooting medium or to the culture medium has been demonstrated to increase nitrogen fixation, biomass production and grain yield. For example, when genistein was added to the rooting medium of soybean nodule number, nodule weight, and nitrogen concentration of plants were increased. Also, in soybean, inoculation of seeds with *Bradyrhizobium japonicum* pre-incubated with genistein increased nodulation at low root zone temperatures (Zhang and Smith, 1995).

Methyl jasmonate is a volatile compound emitted by plant leaves and involved in inter-plant communication, inducing proteinase inhibitor gene expression in neighboring plants (Farmer and Ryan, 1990). However, at sub-micromolar concentrations, they can promote root growth (Tung *et al.*, 1996). Application of JA to leaves also decreases expression of nuclear and chloroplast genes involved in photosynthesis (Creelman and Mullet, 1997). The central role of jasmonates in plant responses to herbivores and defense mechanisms is well investigated. Addition of MeJA initiates *de novo* transcription of genes that are known to be involved in plant chemical defense mechanisms (Gundlach *et al.* 1992). However, with regard to rhizobia, it has been shown that the *nod* genes are inducible by JA and MeJA, as well as by flavonoid inducers (Rosas *et al.* 1998). Some synergistic effects have been observed when two signal inducer molecules were used to pre-incubate cultures of rhizobia subsequently used as inocula. For example, the combination of hesperetin and naringenin resulted in better induction of *nod* gene activities than either hesperetin or naringenin alone (Begum *et al.* 2001).

There is no published work regarding nodulation, N₂ fixation and the resultant growth of bean plants when inoculated with rhizobia pre-incubated with inducer molecules. The work reported here was conducted to evaluate the response of bean plants to *Rhizobium leguminosarum* bv. phaseoli pre-induced with genistein and/or methyl jasmonate.
Materials and Methods
Seeds of common bean (*Phaseolus vulgaris* L.) cultivar Obeto were surface-sterilized using 40% bleach for 1.5 minutes (Montealegre *et al.*, 1995), and then placed in vermiculite filled plastic trays of 27 x 50 x 6 cm, for germination. Four day old seedlings then were transferred to 1-L pots, containing 950 mL of sterilized sand and surface (2:1 v:v) mixture. The experiment was structured following a Randomized Complete Block Design (RCBD) with six replications and two samplings. The plants were grown in a growth chamber with day/night temperatures of 25°C and a 16/8 h photoperiod. Throughout the growth period each pot received 40 mL of Hoagland’s nutrient solution, without nitrogen, (Hoagland and Arnon, 1950) four times a week; the plants were also watered with distilled water if the growth medium appeared dry enough to warrant this.

*Rhizobium leguminosarum* b.v. phaseoli strain 127K105 (Nitragin, Inc. Milwakee, USA) was cultured in TY medium and after 48 h of shaking at 26°C, the bacterial culture was divided into four flasks, each flask representing one treatment. The inoculant treatments were: 1) control, *R. leguminosarum* bv. phaseoli without added inducer, 2) *R. leguminosarum* bv. phaseoli induced with genistein (20 µM), 3) *R. leguminosarum* bv. phaseoli induced with MeJA (50 µM), and 4) *R. leguminosarum* bv. phaseoli induced with both genistein (20 µM) and MeJA (50 µM). The 50 µM concentration was selected for MeJA based on reported ȫ-galactosidase activity of rhizobial strains containing a reporter gene attached to a nod gene promoter - *Rhizobium leguminosarum* bv. ?? in one case (Rosas *et al.* 1998) and *Bradyrhizobium japonicum* strain 532C, in a second case (Mabood, personal communication). After 24 h of further shaking, the inoculants were diluted to an optical density of A 600 0.2 using sterile distilled water (Utlrospec 4300 Pro UV/Visible Spectrophotometer). An OD600 value of 0.08 indicates approximately 10^8 cells mL^-1 (Bhuvaneswari *et al.* 1980). Each seedling received 1 mL of inoculant applied at its base. The plants were seven days old when inoculated.

Leaf nitrogen status was measured indirectly using a SPAD (Soil Plant Analysis Device) leaf greenness meter (SPAD-502, Minolta, Japan). The SPAD data were collected seven days before harvesting. At the end of the experiment, data were collected for the following variables: leaf number, leaf area, shoot and root dry weight, nodule number and weight. The nodule and shoot nitrogen concentration and content of plants were measured using an NC 2500 Elemental Analyzer (CE Instrument Inc., Italy). Nitrogen content data were derived by multiplying dry weight by nitrogen concentration. Shoot and nodule nitrogen content data are given on a per plant basis. The data obtained were statistically analyzed using CoStat software, and means comparisons were conducted using an ANOVA protected LSD (p < 0.05) test. The experiment was repeated twice. The results were similar for both experiments, and the data from the second experiment are shown as an example.

Results and Discussion
Nodule number was increased by application of inducer treatments to the inocula (Table 1). The genistein and MJ treatments led to the initiation of more infections, or the success of a greater number of the initiated infections, leading to the formation of more nodules (Table 1). The utility of flavonoid induction of rhizobial cells in nodule initiation and development are well documented in legume plants such as pea (Bandyopadhyay *et al.* 1996) and soybean (Zhang and Smith, 1995, Pan *et al.* 1998).

The nodule dry weight was not different among treatments, although the genistein plus methyl jasmonate treatment did produce numerical increases that was not statistically significant (Table
1). However, these increases may have been biologically meaningful as nodule dry weight was highly correlated with both plant dry matter and plant nitrogen content (Table 2). However, the inducer treatments did cause more nodules to be formed (Table 2). The same treatments also resulted in a decrease in the average weight per nodule (Table 2), so that plants inoculated with induced rhizobial cells formed more and smaller nodules, with no significant increase in nodule dry weight.

It is known that smaller nodules have greater specific nitrogenase activities than larger nodules (Lindemann and Ham, 1979) and, in our case, the increase in nodule number and associated decrease in nodule size led to an increase in total N present in the plants (Table 1). Plants inoculated with bacteria pre-incubated with genistein and MeJA had the highest per plant nitrogen contents, resulting in an increase of close to 20% over control (Table 1). The pre-induction of the rhizobial nod genes apparently led to more nitrogen being fixed in nodules of plants inoculated with them than with the uninduced control inoculant. Given that the seed used in the experiment were from a relatively uniform seed lot, and were further screened for uniformity by us prior to use, the N content per seed would have varied little, so that differences in total N per plant represents difference in the amount of nitrogen fixed. Thus, treatment of the inocula with inducer compounds led to the formation of more nodules and more efficient nodules, leading to improved N₂ fixation and improved growth. High nodule number and N content observed in this experiment is consistent with the strong correlation observed between the number and percentage of effective nodules produced on bean (Olivera and Graham, 1990). Nodule number per plant is not correlated with N content per plant (Table 2). This is probably because the increased N content follows from greater nodule efficiency, due to the smaller nodule size. In addition, a number of the nodules may have been immature for plants treated with genistein and methyl jasmonate. The higher amounts of nitrogen in plants inoculated with induced rhizobia probably contributed to the greater observed chlorophyll production (as measured by leaf greenness), and, although no data were collected on photosynthetic rates, these were probably increased because dry matter accumulation was increased (Fig. 1). The N content of plants, across all treatments, was highly and positively correlated with plant dry weight (Table 2). This indicates that, when inducers were not added to the applied inocula, bean growth was N limited. Addition of inducers to the inocula increased N fixation, removing the N limitation and resulting in greater plant growth. There were correlations between total N and leaf greenness, and total N and dry matter, suggesting that higher N availability increased the amount of potential photosynthetic activity per unit leaf area (Table 2).

The nitrogen concentrations of bean plants and nodules were not different in present study. The reason for this may have been a dilution effect (dilution of additional N by additional dry matter), as dry matter was increased by inoculation with induced rhizobia (Fig. 1). Pan et al. (1998) observed higher nitrogen concentrations in soybean plants treated with genistein induced rhizobia but, in their experiments, there was very little increase in dry matter production due to the same treatments.

Addition of inducers to the inocula caused both increased growth and accelerated development. Inoculation with R leguminasarum bv. phaseoli cells induced with either MeJA, genistein or both led to a greater leaf number. This may have been due to increased production of bacterium-to-plant signals (lipo-chitooligosaccharides – LCOs) as Atti et al. (2004) have reported that treatment of soybean plants with LCOs led to accelerated flowering in soybean. Atti et al. (2004) also reported that application of LCOs lead to increased plant photosynthetic rates. Thus, application of inducers to R leguminasarum bv. phaseoli cells could have increased bean growth
both through the relief of N limitation to growth and through direct LCO stimulation of plant growth.

There was a measure of synergism between the two inducers as plants inoculated with rhizobia induced with both genistein and MeJA had the highest dry matter accumulation, 14.4% greater than the control plants (Figs 1 and 2).

Following the increases in nodule number, nitrogen contents of plants and dry matter production an increase in seed yield is likely, although these experiments were not allowed to continue all the way to seed development. Shoot dry matter increment, and not just absolute nodulation data, is a key variable in determining final rhizobial effectiveness on bean (MsuMali and Kipe-Nolt, 2002). This has been observed in some legume species but, so far, not in common bean. For example, in soybean nodulation, nitrogen content, and dry matter production have been observed to increase following the application of genistein directly to the root media (Pan et al. 1998). Our results indicate an increase in dry matter that is closely related to changes in plant nitrogen content (Table 2). This could be a way to increase bean dry matter production through improvement of nodulation and nitrogen fixation. The findings reported here, therefore, are unique, suggesting a possible mechanism to address the consistently reported poor nodulation of bean plants under field conditions (Smith and Hume, 1985; Weiser et al. 1985, Park and Buttery, 1989, Graham and Temple, 1984). The data therefore, do not support the suggestion that bean is genetically inferior in N₂ fixation ability (Piha and Muns, 1987).

Another unique aspect of our data is the positive growth response of bean plants to pre-incubation of Rhizobium inoculum with inducer compounds are observed at a temperature of 25 °C, an optimum growth and nodulation temperature for bean (Rubatzky and Yamaguchi, 1997). To our knowledge, Begum et al. (2001) is the most recent publication in this area. They reported of some increases in nodule number and dry matter accumulation in field pea and lentil, but mainly at a lower temperature of 17 °C, when Rhizobium leguminosarum pU1477, and Rhizobium leguminosarum bv. Trifolii 5280 inoculants were pre-incubated with the flavonoid hesperetin. Zhang and Smith (1995) reported higher dry matter production by soybean plants in response to genistein addition to Bradyrhizobium japonicum inoculants at stressfully low root zone temperatures, but not at optimal root zone temperatures. Here, we report a 14.4% increase in dry matter production with consistent increases in nodulation and nitrogen content suggesting that this approach could be utilized in developing a relevant technology to improve yield in bean production. It could also be promising to consider this concept as an area of investigation for yield improvement in other legume crops.

Higher dry weights of plants treated with inducers could be the result of increased nodulation and/or nitrogen fixation by legume plants. The utility of isoflavonoid induction of rhizobial cells in nodule initiation and development are well documented in legume plants such as pea (Bandyopadhyay et al. 1996) and soybean (Zhang and Smith, 1995, Pan et al. 1998). This could be attributed to the higher production of LCO, also known as Nod factor, following nod gene induction. LCO is a host-specific bacteria-to-plant signal molecule essential for the establishment of successful N₂-fixing legume-rhizobia symbioses (Prithiviraj et al. 2003). In the case of R. leguminosarum biovar phaseoli nodC is activated by the constitutively expressed protein coded for by the nodD gene (Davis and Johnston, 1990); expression of nod genes is activated following binding of the nodD protein to an inducer, which allows it to bind to the promoter of the nodABC region. This leads to production and excretion of LCOs. Higher rates of LCO in the rhizosphere may induce the plants to form new root hairs or extra nodule primordial, as has been reported in Phaseolus species (Lopez- Lara et al. 1995).
In conclusion, the data presented indicate that bean plants may not be inferior in nodulation and nitrogen fixation potential. Higher dry matter production, up to 14.4% over control plants, can be achieved through enhancement of the signal exchange between the rhizobia (*R. leguminosarum* biovar *phaseoli*) and the host plant (bean). Genistein and MeJA both induce rhizobial *nod* genes, leading to LCO production, when rhizobial cells are incubated with them prior to the inoculation. It is clear that higher dry matter can be produced by plants inoculated with rhizobia treated with inducer molecules. Thus, these results document the potential role of genistein and MeJA in enhancing bean nodulation, nitrogen fixation and early plant growth.

References


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Table 1: Effect of *R. leguminosarum* bv. *Phaseoli* induced with various inducer signal molecules on plant growth and nodulation variables of common bean (*Phaseolus vulgaris*) plants.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Leaf area per plant (cm²)</th>
<th>Nodule number per plant</th>
<th>Nodule dry weight /plant (g)</th>
<th>Plant N content mg per plant</th>
<th>% Nitrogen increase</th>
<th>SPAD readings</th>
<th>Ind. Nodule dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>719.8</td>
<td>351 c</td>
<td>0.374</td>
<td>126.01 c</td>
<td>---</td>
<td>37.6</td>
<td>1.08 a</td>
</tr>
<tr>
<td>Gen</td>
<td>822.9</td>
<td>457 b</td>
<td>0.375</td>
<td>139.65 ab</td>
<td>10.82</td>
<td>40.4</td>
<td>0.82 b</td>
</tr>
<tr>
<td>MeJA</td>
<td>706.0</td>
<td>480 ab</td>
<td>0.375</td>
<td>138.30 b</td>
<td>9.75</td>
<td>41.8</td>
<td>0.79 b</td>
</tr>
<tr>
<td>Gen+MeJA</td>
<td>814.1</td>
<td>544 a</td>
<td>0.394</td>
<td>151.18 a</td>
<td>19.97</td>
<td>41.45</td>
<td>0.73 b</td>
</tr>
<tr>
<td>Difference</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td>--</td>
<td>ns</td>
<td>***</td>
</tr>
</tbody>
</table>

*ns = Not significant, * = significant at p < 0.05, *** = significant at p < 0.001
Table 2: Correlation coefficients between different aspects of bean plants inoculated with preincubated rhizobia with genistein and methyl jasmonate treatments.

<table>
<thead>
<tr>
<th></th>
<th>Dry weight plant$^{-1}$</th>
<th>Plnat nitrogen content</th>
<th>Leaf No plant$^{-1}$</th>
<th>Nodule No plant$^{-1}$</th>
<th>SPAD</th>
<th>Leaf area plant$^{-1}$</th>
<th>Ind. Nodule dw</th>
<th>Nodule dw plant$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodule N content</td>
<td>46.0**</td>
<td>59.0**</td>
<td>55.0**</td>
<td>29.3$^{ns}$</td>
<td>33.8$^{ns}$</td>
<td>37.2$^{ns}$</td>
<td>2.9$^{ns}$</td>
<td>92.6***</td>
</tr>
<tr>
<td>Nodule dw plant$^{-1}$</td>
<td>79.9**</td>
<td>60.4**</td>
<td>55.0**</td>
<td>29.3$^{ns}$</td>
<td>46.8*</td>
<td>35.0$^{ns}$</td>
<td>1.1$^{ns}$</td>
<td></td>
</tr>
<tr>
<td>Ind. Nodule dw</td>
<td>-6.6$^{ns}$</td>
<td>-12.1$^{ns}$</td>
<td>-46.0*</td>
<td>-89.9**</td>
<td>-40.9*</td>
<td>-25.2$^{ns}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf area plant$^{-1}$</td>
<td>35.6$^{ns}$</td>
<td>22.3$^{ns}$</td>
<td>52.3**</td>
<td>37.2$^{ns}$</td>
<td>17.3$^{ns}$</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SPAD</td>
<td>26.5$^{ns}$</td>
<td>26.8$^{ns}$</td>
<td>51.4*</td>
<td>45.7*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodule No plant$^{-1}$</td>
<td>29.6$^{ns}$</td>
<td>24.0$^{ns}$</td>
<td>61.8**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf No plant$^{-1}$</td>
<td>28.1$^{ns}$</td>
<td>32.9$^{ns}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plnat nitrogen content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>88.8***</td>
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</tbody>
</table>

Figure 1.
Effect of *R. leguminosarum* bv. Phaseoli induced with various inducer molecules on plant dry matter accumulation. Percent increase in dry matter accumulation of common bean plants inoculated with *R. leguminosarum* bv. Phaseoli induced with genistein, methyl jasmonate or both inducers together over control plants receiving *R. leguminosarum* bv. Phaseoli only.
Treatments

Control Gen MeJA Gen+MeJA

Dry weight per plant (g)

2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8

% increase over control

0 2 4 6 8 10 12 14 16 18

a A B c

Treatments

Control Gen MeJA Gen+MeJA

% increase over control

0 2 4 6 8 10 12 14 16 18
Path coefficient analysis for agronomic traits and yield components in rapeseed

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Abstract
Path coefficient analysis was used to investigate the direct and indirect effects of different traits including days to flowering, duration of flowering, days to maturity, plant height, number of pods per main axis, number of pods per plant, length of pod, number of seeds per pod and 1000-seed weight on seed yield of rapeseed (Brassica napus L.). Different breeding lines and cultivars (8 genotypes) and their 56 F₁ generations were evaluated for agronomic traits in this study. The results of path analysis showed that the number of pods per main axis had the maximum and significant, positive direct effect (0.452**) on seed yield. The number of pods per plant and 1000-seed weight also had moderate and positive direct effects on seed yield. Therefore, it seems that the number of pods per main axis and the number of pods per plant can be considered as suitable selection criteria for improvement of seed yield. The direct effect of plant height on number of pods per plant and also the direct effect of days to maturity on pod length and 1000-seed weight were positive and significant. Days to flowering had significantly positive direct effect on days to maturity. The direct effects of flowering duration on plant height and days to maturity were negative and significant (-0.59** and -0.66**, respectively). Therefore, selection based on lower number of days to flowering should improve the early maturity and reduce plant height in rapeseed.

Key words: Path analysis, Rapeseed, Seed yield

Introduction
Rapeseed (Brassica napus L.) is now the third most important oil seed crops in the world (4). Finding of direct and indirect effects of yield components on seed yield can help the breeders to make appropriate selection index and to have a successful breeding program (3). Those yield components that have high heritability and genetic variation and also have genetic relationships with the seed yield can be used as suitable selection criteria (5,6,8,10). Use of simple correlation coefficients could not fully explain the relationships among the traits, however, the path coefficient analysis based on standardized partial regression analysis helps to find the direct and indirect effects of the traits on dependent variable (7). It has extensively been used in breeding programs of different crop species such as Glycin max (2), B. campestris(10), B. juncea(11,13,14,15) and B. napus(1,3). Various studies have been conducted to determine the selection criteria for yield improvement in rapeseed and mustard(3,11,15). Ali (1) reported that there were high genetic and phenotypic variation for plant height and pods per plant in B. juncea and B. napus (1,9,12). High heritability estimates and genetic advance were reported by Singh & Singh (12) for plant height, pods per plant and seed yield in B. juncea L. They also reported that pods per plant, seeds per plant and seed weight had great positive direct effects on seed yield. The objectives of this study were to determine the interrelationship among agronomic traits and yield components of rapeseed.

Materials and Methods
The genotypes of this study consisted of two breeding lines [Yanter ×Tower (BL1), Cobra ×A.W (BL2)], six cultivars of rapeseed (Brassica napus L.) (Shiralee, Regent, Ceres, PF7045/91, Darmor, and Falcon), and their 56 F₁’s generations. The parental genotypes were selected based on their
differences for agronomic traits. The genotypes were evaluated in a simple lattice design with two replications at the Research Farm of Isfahan University of Technology (51°, 32 E longitude, 32 32° N latitude and 1630 m altitude), in the year of 2000. Each plot consisted of three 2-m rows. Between and within row spacing of plants were 60cm and 5cm, respectively. Fertilizers were applied at the rate of 150kg N, 60 kg P2O5 and 75 kg K2O / ha. Two third of nitrogen was used on planting time and the rest applied at the end of rosette stage. The traits of days to flowering, flowering duration, days to maturity, plant height, number of pods per main axis, number of pods per plant, length of pod, number of seeds per pod, 1000-seed weight and seed yield were recorded for each experimental unit. The data were analyzed based on the lattice design and adjusted for the incomplete block effects, when it was needed. The means of 28 crosses (F1’s and their reciprocals) and their parents for each trait were used in calculating correlation coefficients and path coefficient analysis (7).

Results and Discussion
On the basis of physiological relationship of the traits (Figure 1) and path coefficient analysis (Table 1), it was concluded that the number of pods per main axis had significant and positive direct effect (0.452**) on seed yield. The number of pods per plant and 1000-seed weight had also moderate and positive direct effects on seed yield (Table 1). The positive direct effects of pods per plant, seeds per plant and seed weight on seed yield were previously reported in Brassica napus (1,3,7), B. campestris (10, 12) and B. juncea (11,13,14, 15). The direct effect of plant height on the number of pods per plant and also the direct effects of days to maturity on both pod length and 1000-seed weight were positive and significant (Table 2). Duration of flowering had a significant and negative direct effect on both plant height and days to maturity, therefore it seems that selection for higher duration of flowering can be considered as indirect selection for reduction of plant height and early maturity in rapeseed. Also, it can be concluded that the number of pods per main axis and finally the number of pods per plant which had positive direct effects and significant positive correlation with seed yield, can be considered as suitable selection criteria for improving the seed yield of rapeseed.

References

Figure 1: Path diagram for the traits in rapeseed.
Table 1: Direct (on diagonal) and indirect (off diagonal) effects of yield components on seed yield of rapeseed.

<table>
<thead>
<tr>
<th>Traits</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Correlation coefficient with seed yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Number of pods per main axis</td>
<td>0.452**</td>
<td>0.12</td>
<td>-0.008</td>
<td>0.023</td>
<td>0.045</td>
<td>0.63**</td>
</tr>
<tr>
<td>2- Number of pods per plant</td>
<td>-0.006</td>
<td>0.248</td>
<td>0.038</td>
<td>-0.025</td>
<td>-0.30</td>
<td>0.49**</td>
</tr>
<tr>
<td>3- Pod length</td>
<td>0.025</td>
<td>-0.014</td>
<td>-0.128</td>
<td>0.108</td>
<td>-0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>4- Number seeds per pod</td>
<td>0.056</td>
<td>0.051</td>
<td>-0.074</td>
<td><strong>0.188</strong></td>
<td>-0.064</td>
<td>0.16</td>
</tr>
<tr>
<td>5-1000-seed weight</td>
<td>0.089</td>
<td>-0.028</td>
<td>0.016</td>
<td>-0.053</td>
<td><strong>0.226</strong></td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 2: Direct effects of phenological traits on other traits and yield components in rapeseed.

<table>
<thead>
<tr>
<th>Direct effect of days to flowering on</th>
<th>Flowering duration</th>
<th>Days to maturity</th>
<th>Plant height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.29</td>
<td>0.41*</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Direct effect of flowering duration on</th>
<th>Days to maturity</th>
<th>Plant height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.66*</td>
<td>-0.59**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Direct effect of days to maturity on</th>
<th>Pod length</th>
<th>Number of seeds per pod</th>
<th>1000-seed weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.48**</td>
<td>0.31</td>
<td>0.48**</td>
</tr>
</tbody>
</table>
Nuclear techniques aided studies for sustainable biomass production in salt affected soils and using haloculture method

J.Rastegari¹, H.Abbasalian¹

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Abstract
More than 25 Mha of salt affected land exists in Iran. Considering that about 90% of the country is arid, the fresh water resources are limited. Population growth of the country and the consequent increase in food demands, makes utilization of saline land and water resources for increased food production and socio economic improvement of the salt-affected areas, an important task. Haloculture can offer an economic and practical alternative towards achieving these goals. Haloculture is economical production of highly salt tolerant plants and halophytes in highly saline lands with saline irrigation water. The main objective of this study is to evaluate the adaptability of several local and imported salt tolerant plants with harsh environmental conditions of Iran. Other objectives of this project include utilization of Isotope techniques for irrigation scheduling of plants, and hydrological evaluation of underground water resources.

Chah Afzal area in Yazd province was selected as experimental site. 21 species were selected for adaptability study. More than 10000 seedlings of the salt tolerant plants were produced, and planted in 1999 at the experimental site. Before plantation, soil samples were collected and analyzed. Three (2002) years later, again the soil samples were collected and analyzed to evaluate the changes in soil chemical properties. Survived plant populations were counted 1 and 2 years after plantation, and their percent of survival rates were calculated. By the use of neutron meter, irrigation scheduling for pistachio, pomegranate, Eucalyptus, Atriplex, Acacia, Haloxylon, Tamarix, Kochia, wild olive, and Sesbania was performed. Also, by the use of Isotope Hydrology technique hydrological features of the underground water resources were evaluated.

The results showed that out of 21 selected salt tolerant plants, 12 species, and sub-species performed adequately. These species have potential uses for orchard products (Pistachio, Pomegranate, and wild olive), for forage (Acacia, Atriplex, Sesbania, Kochia and Kallar grass), and for wood (Eucalyptus, wild olive, Haloxylon and Tamarix). Plantation of salt tolerant species and irrigation had profound effect on reduction of soil PH, EC, and SAR. Isotope hydrology results indicate that most of the recharge water of Chah Afzal water basins originates from precipitations in Shir Kooh mountain range, and they are less than 50 years old. Due to high evaporation in the regions, and passage through different geological deposits, water, contain high concentration of different salts, and thus are saline.

In general, the results of this project revealed that haloculture can be utilized effectively in Iran to produce different salt tolerant species with different economical potentials. It is recommended that to continue further experiments and studies on these successful plants in a larger scale, for economical production of forage, food, feed, and wood.

Keywords: Nuclear techniques, Neutron probe, Soil salinity, Isotopes, Salt tolerance

Introduction
Salt-affected soil is wide spread in arid and semi arid regions of the world. There are 380 million hectares of saline soil on the earth. Reclamation of salt affected land is being practiced using different combinations of leaching and drainage, beside the high cost of these work, this solution has some other difficulties such as removal of drained water. One more effective way of using saline land and saline water is to use plants. Plants have vast genetic variability and during their evolution they have adapted to so many kinds of habitats. A Large
number of crop plants, vital to human life as food, feed and fiber sources, trace their origins to the part of the Old World known as the Middle East, and a good number have their center of origin and center of diversity in or around Iran (1, 2, 3, 4).

Iran is located between 25 and 40 North latitude and is predominately an arid and semi-arid country. It covers 165 Mha, out of this area 25 Mha is highly affected by saline soil. According to data published by UNESCO, IRAN ranks 3rd in Asia only after Russia and China and ranks fifth in the world in total salt affected land areas. The demonstration site is located at Yazd province in central Iran. Yazd climate is harsh dry and desert-like. Annual rainfall is low (60-80mm) and the evaporation is very high (4000mm) due to rather low humidity and hot weather with great day and night temperature fluctuations. Thereby the province has the worst peculiarities of the word. Annual temperature ranges from – 16 to 45°C. About 90% of the land surface are heavily affected by salinity.

Material and Methods

In order to perform management practices to select the best salt-tolerant crops and lower salt accumulation in top soil, 10000 pots, of different salt-tolerant species both local and from Pakistan (see the list on next page) were sowed in the green house of Nuclear Research Center for Agriculture and Medicine (Karaj). These seedlings were grown to 8 inches tall and were transplanted to the site. In Feb. 1999. A number of seedlings were also obtained from the nursery of natural resources organization, and the total of 21 salt–tolerant species were selected to test in the site of Yazd. Survival rate of different salt tolerant species are presented in table 5.

Ten hectares of the land of Chah- Afzal salinity research station were devoted to this activity. It is worth to mention that the land was divided to plots, with area about 5000m² surrounded by Tamarix trees. Soil characteristics of the experimental site are given in table 2 and quality of water available for irrigation is presented in table 1.

In order to measure soil water content, monitoring its changes with time and make suitable schedules for irrigation, ten access tube ( in two replications ) were installed up to about 180cm soil depth, in 5 selected plots (Those could resist and tolerate against highly salt accumulation and severe condition of Yazd i.e Haloxylon aphyllum, Elaeagnus angustifolia, Pistacia Vera, Eucalyptus camaldulensis and Atriplex conescens ).The neutron probe CPN 503DR calibrated and have been used for irrigation scheduling. Irrigation was started when the soil water depletion was about 50% of volumetric soil water content at F.C. Amount of irrigation water calculated based on difference between soil moisture prior to irrigation and the soil moisture at field capacity.

In order to compare salt tolerance of different species and for their ability to reduce soil salinity, one hectar plots of different species were considered. Soil salinity was measured twice (before planting and after 3 years later. For studying the dynamics of underground water via isotope analysis methodology , some water samples were collected at regular intervals from the site and from several wells located 1-2 Km around the site. The samples were analyzed at PINSTEC, Islamabad- Pakistan.

Results

The results of soil EC, PH and SAR changes are given in Fig1,2,3. Fig1,3 illustrate electrical conductivity and sodium absorption ratio at upper layer of soil profile have decreased due to leaching of sodium salts and chloride ions.Fig2 demonstrates that soil PH has been improved. This attributes to cultivation of salt tolerance species which added organic matter to the soil.Organic acids, the by product of O.M biological decomposition lowered the soil PH.

Discussion
In order to screen among salt-tolerant species for the local conditions, adaptation trials are necessary. Such trials were successfully implemented in this project. The site served very well to demonstrate the capabilities and usefulness of Biosaline Agriculture to local farmers and research scientists. The application of neutron probe as a means of nuclear technique for irrigation management was also successfully demonstrated. However, due to its high cost, and the high level of expertise required for operation, it can be used mainly by research scientists and research institutes as a reliable tool for irrigation management studies.

Based on the results of this adaptation trial study, *Punica granatum*, *Elaegnus angustifolia*, *Acacia ampliceps*, *Pistacia Vera*, *Eucalyptus camaldulensis*, *Haloxylon aphyllum*, *Tamarix* and *Atriplex* were the most suitable salt-tolerant species among the ones tested for the local conditions. However, more salt-tolerant species should be trialed. The appropriate agronomic practices for maximum production of these species need to be studied further.

Results of neutron meter study revealed that *Eucalyptus* and *Sesbania* consumed 2.5 times more water than *Pistachio*, wild olive, *Atriplex*, *Haloxylon*, *Tamarix*, *Acacia* and *Kochia*, and 2 times more than pomegranate. Plantation of species with low water requirement for arid region of Chah Afzal is more desirable (fig 4).

**References**


**Table Legends**
Table 1. Quality of water available for irrigation at the selected site of Yazd during Feb 1999 till Oct 1999
Table 2. Soil characteristics of the selected site of Yazd at initial stage
Table 3: Some of the soil characteristics 3 years after cultivation of salt tolerance species at 0-30 cm depth
Table 4: List of plant species cultivated in Chah Afzal Research station (1999-2002)
Table 5: Survival rate of the salt-tolerant species cultivated at CASRS, Yazd, Iran

**Figure Legends**
Fig 1: EC changes
Fig 2:PH changes
Fig 3:SAR changes
Fig 4:Water application pattern

Table 1. Quality of water available for irrigation at the selected site of Yazd during Feb.1999 till Oct. 1999
### Table 2. Soil characteristics of the selected site of Yazd at initial stage

<table>
<thead>
<tr>
<th>Depth</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-35cm</td>
<td>35-70cm</td>
</tr>
<tr>
<td>EC</td>
<td>ds/m</td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>P.W.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>S.A.R.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E.S.P.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>E.S.R.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Texture</td>
<td>L</td>
<td>S.L.</td>
</tr>
</tbody>
</table>

P.W. = Soil moisture  
S.A.R. = Sodium Absorption Ratio  
E.S.P. = Exchangeable Sodium Percentage (\(\frac{Na}{CEC} \times 100\))  
S.P. = Saturation percentage  
E.S.R. = Exchangeable Sodium Ratio (\(\frac{Na}{CEC-\text{Na}}\))  
R.S.C. = Residual Sodium Carbonate  
S.I. = Saturation Index= 8.4 – (pK₂-pKsp) + p (Ca + Mg) + P (CO₃ + HCO₃)  
S.A.R. adj= S.a.r.[1.0+(8.4-pHc)]

### Table 3: Some of the soil characteristics 3 years after cultivation of salt tolerance species at 0-30 cm depth

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>pH</th>
<th>EC(\times 10^3)</th>
<th>SAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punica grantum</td>
<td>8.1</td>
<td>9.1</td>
<td>3.47</td>
</tr>
<tr>
<td>Elaeagnus angustifolia</td>
<td>8.03</td>
<td>8.88</td>
<td>12.89</td>
</tr>
<tr>
<td>Acacia ampliceps</td>
<td>8.12</td>
<td>9.65</td>
<td>12.75</td>
</tr>
<tr>
<td>Pistacia vera</td>
<td>8.2</td>
<td>12.7</td>
<td>21.78</td>
</tr>
<tr>
<td>Atriplex lentiformis</td>
<td>8.2</td>
<td>11.87</td>
<td>20.319</td>
</tr>
<tr>
<td>Atriplex halimus</td>
<td>8.2</td>
<td>15.81</td>
<td>24.28</td>
</tr>
<tr>
<td>Haloxylon aphyllum</td>
<td>8.04</td>
<td>30.4</td>
<td>53.92</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>7.8</td>
<td>8.68</td>
<td>13.3</td>
</tr>
<tr>
<td>Tamarix</td>
<td>8.08</td>
<td>10.8</td>
<td>16.8</td>
</tr>
</tbody>
</table>

### Table 4: List of plant species cultivated in Chah Afzal Research station (1999-2002)

<table>
<thead>
<tr>
<th>Plant Species</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Punica grantum</td>
<td>12-</td>
<td></td>
</tr>
<tr>
<td>2- Elaeagnus angustifolia</td>
<td></td>
<td>Atriplex lentiformis</td>
</tr>
<tr>
<td>3- Fraxinus oxyearpa</td>
<td>14-</td>
<td>Atriplex canescens</td>
</tr>
</tbody>
</table>

3- Fraxinus oxyearpa  
14- Atriplex canescens
4- Olea europaea L.  
5- Ulmus minor  
6- Ailanthus glandulosa  
7- Robinia pseudoacacia  
8- Eucalyptus camaldulensis  
9- Pistacia vera L.  
10- Acacia amplus  
11- Atriplex numularia  
15- Haloxylon aphyllum  
16- Tamarix ramosissima  
17- Tamarix aphylla  
18- Wheat (13 varieties)  
19- Barley (Three varieties)  
20- Triticale (Three varieties)  
21- Rye (Three varieties)

Table 5: Survival rate of the salt-tolerant species cultivated at CASRS, Yazd, Iran

<table>
<thead>
<tr>
<th>Plant name</th>
<th>No. of Seedlings Planted</th>
<th>% Survival 1 year after plantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punica grantum</td>
<td>612</td>
<td>49</td>
</tr>
<tr>
<td>Elaeagnus angustifolia L.</td>
<td>357</td>
<td>70</td>
</tr>
<tr>
<td>Acacia amplus</td>
<td>463</td>
<td>66</td>
</tr>
<tr>
<td>Pistacia vera L.</td>
<td>208</td>
<td>92</td>
</tr>
<tr>
<td>Eucalyptus camaldulensis</td>
<td>259</td>
<td>39</td>
</tr>
<tr>
<td>Haloxylon aphyllum</td>
<td>408</td>
<td>86</td>
</tr>
<tr>
<td>Tamarix</td>
<td>340</td>
<td>88</td>
</tr>
<tr>
<td>Atriplex</td>
<td>1020</td>
<td>87</td>
</tr>
</tbody>
</table>

Fig 1: EC changes

- before cultivation
- after cultivation
Proceedings of The Fourth International Iran & Russia Conference

**Fig 2: PH changes**

<table>
<thead>
<tr>
<th>Species</th>
<th>PH before</th>
<th>PH after</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punica grantum</td>
<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
<td>Elaeagnus angustifolia</td>
<td>8.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Acacia</td>
<td>8.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Pistacia vera</td>
<td>8.3</td>
<td>8.2</td>
</tr>
<tr>
<td>Atriplex lentiformis</td>
<td>8.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Atriplex halimus</td>
<td>8.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Haloxylon aphyllum</td>
<td>8.6</td>
<td>8.5</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>8.7</td>
<td>8.6</td>
</tr>
<tr>
<td>Tamarix</td>
<td>8.8</td>
<td>8.7</td>
</tr>
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</table>

**Fig 3: SAR changes**

<table>
<thead>
<tr>
<th>Species</th>
<th>SAR before</th>
<th>SAR after</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punica grantum</td>
<td>450</td>
<td>440</td>
</tr>
<tr>
<td>Elaeagnus angustifolia</td>
<td>430</td>
<td>420</td>
</tr>
<tr>
<td>Acacia</td>
<td>440</td>
<td>430</td>
</tr>
<tr>
<td>Pistacia vera</td>
<td>450</td>
<td>440</td>
</tr>
<tr>
<td>Atriplex lentiformis</td>
<td>460</td>
<td>450</td>
</tr>
<tr>
<td>Atriplex halimus</td>
<td>470</td>
<td>460</td>
</tr>
<tr>
<td>Haloxylon aphyllum</td>
<td>480</td>
<td>470</td>
</tr>
<tr>
<td>Eucalyptus</td>
<td>490</td>
<td>480</td>
</tr>
<tr>
<td>Tamarix</td>
<td>500</td>
<td>490</td>
</tr>
</tbody>
</table>

**Fig 4: Water application pattern**

<table>
<thead>
<tr>
<th>Water applied(m3/ha, yr)</th>
<th>No of irrigation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10000</td>
</tr>
<tr>
<td>2000</td>
<td>8000</td>
</tr>
<tr>
<td>4000</td>
<td>6000</td>
</tr>
<tr>
<td>6000</td>
<td>4000</td>
</tr>
<tr>
<td>8000</td>
<td>2000</td>
</tr>
<tr>
<td>10000</td>
<td>0</td>
</tr>
</tbody>
</table>

Species:
- Eucalyptus
- Sesbania
- Aculeata
- Pistacia
- Vera
- Elaeagnus
- Angustifolia
- Atriplex
- Aphylla
- Aphyllum
- Atriplex
- Haloxylon
- Aphyllum
- Acacia
- Kochia
- Indica
- Punica
- Granatum
Molecular tracing of the protein kinase and prosystemin genes expressed in the plants of hypersensitive tobacco Samsun NN during an induced antiviral resistance

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¹ Institute of Biochemistry and Genetics, Ufa, Russia. Phone: +8-3472-356088 Email: rozhnova@mail.rb.ru; ² Vavilov Institute of General Genetics, Moscow, Russia. Phone: +8-3472-135-11-51 Email: pukhalsk@vigg.ru

Abstract

One of the basic tasks of genetics of plant immunity is the research of differences total and locus-specific of expression spectra at hypersensitive tobacco Samsun NN in leave tissues with local and systemic resistance and reason of their occurrence. In our project the molecular analysis of the mechanisms of antiviral induced resistance at hypersensitive tobacco Samsun NN was carried out with application of modern methods of molecular biology: DDRT-PCR (differential RNA display), 3’-RACE technique (Rapid Amplification of cDNA 3’-ends) and cDNA-AFLP. In molecular genetic researches of 2001-2003 years we found out two prospective markers of antiviral induced resistance expressed in lower leaves and five prospective markers expressed in top systemic leaves of tobacco. Three out of five assumed markers systemic resistance created due to antisense primers of the locus SR160 (systemin receptor of kinase nature) were cloned in T/A a vector pGEM®-T Easy (Promega, USA) and then are transformed into E. coli XL-1 Blue cells. The samples were sequenced at automatic ABI Prism 310 Genetic Analyzer. Sequenced DNA probes were compared to the data of GenBank, utilizing BLASTN, BLASTX and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/). Probe RSR160-2a (285 pairs of nucleotides) had not a homology with known sequences. Probe RSR160-3a (307 pairs of nucleotides) had a high homology with human DNA (more than 95%). Probe RSR160-4a (245 pairs of nucleotides) had a weak homology with mRNA of systemin receptor SR160 Lycopersicon peruvianum (23 out of 23 nucleotides, 22-aggaccgaagaagaaaagaa-43) and with unknown chromosome sequence of Mus musculus (22 out of 22 nucleotides). Obviously any of probes had a sufficient homology with already known DNA sequences. The results of the present work will ensure the transferring to cloning of full-size genes - candidates and their function analysis. This work was supported by the Russian Foundation for Basic Research, project nos. 01-04-48706 and 01-04-49272.

Key Words: hypersensitive tobacco Samsun NN, protein kinase, prosystemin, SAR – systemic acquired resistance, TMV – tobacco mosaic virus

Introduction

Signaling systems play a vital role in the plant cell-cell communication (Tarchevskiy, 2000). Intensive investigations are focused on the plant mitogen-activated protein (MAP) kinase signaling cascades (Tena et al., 2001) and systemin signaling systems of plants (Ryan et al., 2002). It is known protein kinases as the central elements of signal transduction are able activate
of the transcription factors and others effectors in a cell. The important role in the initiation of protective reactions of plants belongs to protein kinases forming the receptor structures on a surface of a cell to the signal molecules of protein nature. The polypeptide molecules also are the important receptor intercellular signals (phytohormones) regulating protective and ontogenetic processes at plants. However some polypeptide signal molecules are identified (Ryan et al., 2002). To the present time the genes of prosystemin system of the two types are identified. They are tomato-like system (also isolated from potato, bell pepper and black nightshade) and tobacco-like system (isolated from tobacco only). However both systemin signaling systems functioning at Solanaceae have not nucleotide homology at all. To the present time there are many facts showing of an opportunity of modulation (activation or inhibition) of one signal system by means of the intermediate products (of the secondary intermediaries) of other systems (Tarchevskiy, 2000). However interaction of such signal systems as protein kinase and prosystemin systems of plants are investigated weakly. More recently the convergence of the different signal ways at Lycopersicon peruvianum induced by systemin, oligosaccharide elicitors and ultraviolet at a level of MAP-kinases was shown (Holley et al., 2003). On the other hand special function importance of a systemin receptor SR160 of kinase nature is due to wide specificity. Systemin and also brassinolides are able to use this surface receptor SR160 in signaling (Szekeres, 2003).

Before we reported about antiviral immunostimulant effects of emistim (Rozhnova et al., 1999), arachidonic acid (Rozhnova et al., 2001), ubiquinone 50 (Rozhnova and Gerashchenkov, 2002) and vitamin E (Rozhnova and Gerashchenkov, 2004). As we have found out, formation of SAR induced by plant viruses and elicitors are accompanied dramatically changes of proteins (Rozhnova et al., 2001; 2003, 2004). Apparently, specific changes of some expressed genes and signal systems would be correspond to these biochemical changes. Our purpose was to search and analysis of signal transduction genes (protein kinase and prosistemin genes in particular) differently expressed at hypersensitive tobacco during formation of systemic antiviral resistance.

Materials and Methods

6-8 week-old virus-free hypersensitive tobacco plants (Nicotiana tabacum L., cv. Samsun NN) were treated by elicitors (immunization) or ordinary strain of TMV (vaccination) as is described in our works (Rozhnova et al., 1999; 2001; 2002; 2004). RNA samples were isolated from the lower and upper leaves at different time points (from 10 to 100 minutes with the 10 minutes step, 2, 3 and 3 hours) after TMV treatment. cDNA synthesis was carried out in 30 mkl using of a special ATtract Series 9600 mRNA Isolation and cDNA Synthesis Systems kit (Promega, USA) as it is described in (Gerashchenkov and Rozhnova, 1999). Totally more than 100 mRNA samples have been analysed in the work.

We have used motif-directed differential RNA display and RACE-3’ (Rapid Amplification of cDNA 3’-ends). Our approach included: 1) selection of biotinilated cDNAs on paramagnetic beads instead of DNAse treatment of RNA samples and 2) applications of motif-directed oligos (17-26 bases in length) homologous to the sequences of protein kinase and prosistemin genes. The used primers are given in the Table. T-anchor primers with 2 and 3 additional nucleotides on 3’-end were used as reverse primers.
Results
We found out two prospective markers of antiviral induced resistance expressed in lower leaves and five markers expressed in top systemic leaves of tobacco at the different time steps. Some molecular genetic markers are shown at Fig.1 and Fig.2. Northern hybridization assay of screened mRNAs with verified markers and an actin probe (as a positive control) confirmed the differential expression of the markers (results are not shown). The expression patterns of RALF (Rapid Alkanization Factor), Tob Sys I and Tob Sys II of tobacco-like systemin genes were simultaneously investigated. However co-expressions of RALF genes and isolated markers of resistance was not detected.

Tested markers of systemic antiviral resistance related to the locus SR160 were cloned and sequenced. Isolated DNA sequences were legated in T/A a vector pGEM® -T Easy (Promega, USA) and then are transformed into cells E. coli XL-1 Blue. Samples were sequenced at the automatic Sequencer ABI Prism 310 Genetic Analyzer. Sequenced DNA probes were compared to the data of GenBank, utilizing BLASTN, BLASTX and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/). Probe RSR160-2a (285 pairs of nucleotides) had not a homology with known sequences. Probe RSR160-3a (307 pairs of nucleotides) had a high homology with human DNA (more than 95 %). Probe RSR160-4a (245 pairs of nucleotides) had a weak homology with mRNA of systemin receptor SR160 Lycopersicon peruvianum (23 out of 23 nucleotides, 22-aggacggaagagaagaaaagaa-43) and with unknown chromosome sequence of Mus musculus (22 out of 22 nucleotides). Obviously any of probes had a sufficient homology with already known DNA sequences.

Discussion. We have shown some biochemical changes in tobacco and potatoes plants at the formation of antiviral resistance (Rozhnova et al., 2001; 2002; 2003; 2004). However the comprehension of molecular mechanisms underlying antiviral resistance in plants is impossible without identification of key genes supervising of the signal transduction. That is why the genes of plant signaling (protein kinase and prosystemin in particular) were in focus of our investigation.

One of the general tasks of the plant immunity genetics is the analysis of differences in total and locus-specific expression spectra of hypersensitive tobacco Samsun NN at the local and system resistance. Essential differences in molecular spectra were not revealed at the expression analysis of the tomato-like prosystemin. The insignificant polymorphisms were due to minor components in spectra. Moreover any of amplicons was common for the immunization or TMV vaccination. The reason of low heterogeneity of the expression spectra in the leaves of Nicotiana tabaccum L may be due to absence of the tomato-like prosystemin. The point is that systemin genes of tobacco differ from similar genes of a tomato-type; they have no any nucleotide homology (Pearce et al., 2001). The American researchers have found out the tomato-like systemin in potatoes, pepper and other species of Solanaceae, but not are in the tobacco. Probably, the tomato-like prosystemin spectra in tobacco leaves obtained in our work reflect the existence of certain common nucleotide sequences (look like pseudo-gene), which have no immediate attitude to the formation of antiviral induced resistance in of tobacco (Scheree et al., 2003). In 2002 we took into account the data kindly given by Clarence A. Ryan and our researches were directed on the investigation of the genes underlying the tobacco-like prosystemin signaling, in particular, genes of the peptide precursor Tob Sys I and Tob Sys II tobacco-like prosystemin and protein kinases SR160. On the basis of new designed oligos ## 6-10 (see Table), the analysis of expression polymorphism of the potential systemin receptor SR160 of kinase nature and tobacco-
like pro-systemin locus including the genes Tob SysI and Tob SysII was carried out. We was able to detect temporal changes in the expression spectra for the pro-systemin locus in a temporary interval from 10 to 100 minutes with a 10 minutes step in the lower leaves with local resistance and the SR160 locus after TMV inoculation in the upper leaves with system resistance. Thus, 2 markers were detected using the primers to the tobacco-like pro-systemin locus. One of such markers is given on Fig.1. 3 potential markers of induced resistance to TMV infection in hypersensitive tobacco plants in a temporary interval from 100 minutes till several hours were found. One of such markers is shown at Fig2. As it following from the literature data, the RALF locus is expressed simultaneously with Tob Sys I and Tob Sys II genes of tobacco-like systemin (Pearce et al., 2001). In our work co-expression of RALF locus and isolated markers was not detected. Sequencing and comparative analysis of RH-ccc of probes using the program BLAST have confirmed an absence of the essential homology of isolated probes with known DNA sequences. However we shall note the marker RSR160-4a (size 245 nucleotides) had a weak homology with mRNA of systemin receptor SR160 at \textit{Lycopersicon peruvianum}.

Conclusion.

Probably the results demonstrating extremely low level of polymorphisms in expression spectra of tomato-like pro-systemin and protein kinases genes at tobacco prove obliquely confirmation of results obtained by lab of Clarence A. Ryan who have shown absence of tomato-like systemin activity in the suspension culture of tobacco (Ryan et al., 2002). The executed researches directed to the isolation of common DNA amplicons expressed in systemic leaves of tobacco have allowed revealing five molecular markers of antiviral resistance. Three out of five markers were sequenced. However all three of SR 160 markers had no an essential homology with already known DNA sequences. The truth, the cloned and sequenced probe RSR160-4a (size 245 nucleotides) had a weak homology with mRNA of the systemin receptor SR160 from \textit{Lycopersicon peruvianum} (23 out of 23 nucleotides, position \texttt{22-aggacgaggaagaaaaaga-43}).

We are going to carry out the cloning of full genome candidate genes and their function analysis. The results of the present work will useful for the creation of gene engineering constructs to produce transgenic plants carrying the genes supervising the formation of systemic resistance against broad viral pathogens.

Acknowledgements. We thank PhD Sang-Gu Kang who has presented an actin probe. This work was supported by Russian Foundation for Basic Research (grants N 01-04-48706 and N 01-04-49272).

References


Pictures and Photos

Fig. 1.

<table>
<thead>
<tr>
<th>Lower leaves</th>
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<td>60 70 80 90 100</td>
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Fig. 2.
Figure Legends

Fig.1. Expression pattern at the lower leaves with local resistance and upper leaves with system resistance. Differently expressed amplicons were detected 90 and 100 minutes after TMV treatment of lower leaves.

Fig.2. Expression pattern of SR160 locus at the upper leaves with the systemic antiviral resistance. The bands arrowed are markers of the antiviral resistance.

Tables Legends

Table. The set of primers

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<td>5’-ggc-gaa-ggc-aca-tac-gg-3’</td>
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<td>3</td>
<td>5’-gcc-aga-tgg-tat-gaa-tgg-3’</td>
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<td>4</td>
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<td>10</td>
<td>R-SR160 5’-agc-ctg-caa-gta-cat-caa-ctc-3’</td>
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Role of Endophytic Fungi in Forage Production of Tall Fescue, Festuca arundinacea

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Abstract
Symbiotic relationship has been found between endophytic fungi and most cool-season grasses including 80 genera and 100 species of subfamily Pooideae. In this relation, endophytic fungi gain their food and energy from host plants and instead improve host characteristics such as yield and resistance to intense grazing and biotic and abiotic stresses. These effects induced from endophytic fungi can increase net production of plant forage. Six genotypes of tall fescue, Festuca arundinacea, were used in this research to evaluate endophytic fungi role in forage production. Endophyte-free versions of each genotype were prepared using a fungicide mixture of Fulicor and Propiconazol from endophyte-infected plants. These genotypes were planted in a randomized complete block design with three replications in the field. Fresh and dry weight of forage produced, tiller number and rate of re-growth of each genotype (endophyte-infected and endophyte-free versions) were measured after eight months. Results of this study showed that endophytic fungi could increase fresh and dry weight of plant forage. Endophyte-infected plants had two to ten times more tiller number than endophyte-free counterparts. Endophyte also enhanced re-growth of infected plants after clipping. This may be due to allocation of more assimilates to plant roots. This study showed that endophytic fungi can improve production of plant forage and may be used in other grass species important for forage production.

Key words: endophyte, Festuca arundinacea, forage plant, Neotyphodium

Introduction
Tall fescue (Festuca arundinacea Schreb.) is infected by the fungus Neotyphodium coenophialum Glenn, Bacon, Hanlin. This fungus spends its life cycle within the plant without any external sign of infection (Siegel et al., 1985). Hyphae of Neotyphodium are distributed in all plant parts except plant roots (Bacon et al., 1977; Clark et al., 1983). This association of tall fescue and its endophyte has been suggested to be a mutualistic symbiosis in which, grass benefits by increased growth, deterrence of insects and mammalian herbivores, and tolerance to stress environments while the fungus receives nutrients from the plant apoplast, reproduce and disseminate via seed production (Bacon and Siegel, 1988). Endophyte infection has increased tillering and herbage growth in tall fescue clones (Belesky et al., 1987). At the population level, infected tall fescue seedlings, showed greater germination and tiller and dry weight production than noninfected counterparts (Clay, 1987). Results on the effect of endophyte on these traits in the field, however, are not crucial and involve some contrasting reports. Siegel et al. (1984) found no difference in survival rate and herbage yield between infected and noninfected populations under well adapted condition. By contrast, in stressful environment, Read and Camp (1986) reported enhanced growth and better survival of infected plants. The objective of this study was to determine the effects of the
endophyte association on forage production and associated traits in a broader range of tall fescue genotypes.

Materials and Methods

Plant Materials
Six tall fescue genotypes clonally propagated were used for this study. Seeds of three accessions were originally planted in the greenhouse and six compatible host-endophyte combinations from accessions were selected. Microscopic examination of leaf sheaths confirmed infection of plants and compatible combination was chosen based on high hyphae concentration and its unbranched hyphae movement. Each selected plant was separated into two groups of individual tillers which were transplanted into separate plots in the field. One plot of each plant (genotype) was treated (sprayed) with Propiconazole [1-(2-(2-4-dichlorophenyl)-4-propyl-1, 3-dioxolan-2-y1) methyl-1H-1, 2, 4-triazole] and Folicur as a fungicide mixture at 2a.i. (active ingredient) and 1ml per liter, respectively. The fungicide treatment was repeated two times, 7d apart. Microscopic examination of new tillers produced in the treated plots confirmed eradication of the endophyte. New tillers of endophyte-infected and endophyte-free plants were transplanted to experimental field. The experimental design was a randomized complete block with a factorial arrangement of the treatments (six genotypes and their infection status) with 3 replications including 6 hills of plant per replication. Each hill comprised of approximately 5 tillers. Plots were 1.5 × 1.5 m² in size and contained a rich clay-loam soil. Throughout the experiment, all plots were watered twice a week, and fertilized (75 kg/ha N) before flowering stage in the spring.

Plant Analysis
After 8 months of field growth, plants were cut from 5 cm of ground level, oven dried at 60°C for 48 h and weighted. Shoot fresh and dry weight of one hill was also measured. One hill from each plot was randomly selected, and number of tillers per hill was measured. After that, the hill was washed free of soil, roots were separated, weighted and oven dried at 60°C for 48 h and weighted again to measure fresh and dry matter of root. After two weeks of cutting, the height of regrowth in each plot was measured. Analyses of variance were performed for each variable and treatment means were compared using Duncan’s multiple range test.

Results
The experimental plant genotypes used in this study showed significant (P<0.01) differences for all variables. When pooled across genotypes, the variables including shoot fresh and dry weight, root fresh and dry weight, plot herbage yield, numbers of tillers per hill and regrowth height after cutting were significantly (P<0.01) increased by endophyte infection (Figure 1) but the increase rate among genotypes was different and in some cases endophyte-free genotypes had greater value of the trait than their endophyte-free counterparts. This was resulted in significant (P<0.01) interaction between genotype and endophyte infection for shoot and root weight (fresh and dry), numbers of tillers per hill and regrowth height but not for plot herbage yield (Table 1).

Discussion
Tiller number per hill, herbage growth, and root fresh and dry yield were increased in infected clones (Fig. 1). Similar results in tillering and herbage production have been obtained with infected and noninfected clones of tall fescue and perennial rye grass (De Battista et al., 1990; Arechavaleta et al., 1989; Belesky et al., 1987). Infected
plants produced (P<0.01) more root dry matter than endophyte free counterparts. Enhanced root mass was also reported in endophyte-infected clones of perennial ryegrass (Latch et al., 1985). In this study, it is showed that endophyte infection may decrease growth of at least some tall fescue genotypes, but it is not clear how the endophytic fungus may alter the plant’s physiology to achieve these differences. Change in hormonal balance of the host plant could be a possible fungal mechanism to alter host growth (Arachevaleta et al., 1989; Belesky et al., 1987). It has been established that the endophyte of tall fescue produces IAA in culture (De Battista, 1989), But, the effect on IAA in the plant are still unknown.

The high interactions of endophyte infection and plant genotype may result from specific relationships between each plant and fungus genotype. This effect implies genotypic differences among individuals of both plant and fungus. Greater regrowth after cutting in endophyte-infected plants, suggests that endophyte infected plants were more efficient in using supplies of root for shoot regrowth. This effect may also relate to alteration in plant physiology and hormonal balance.

In summary, endophyte infection may increase root growth and enhance forage production of tall fescue host plant, but because of the presence of interactions between endophyte and grass genotypes, the same effect may not be extrapolated to other genotypes of the endophyte–harboring plant species. More information is needed on the physiological bases and mechanisms by which fungal endophyte affect host growth characteristics, along with the effect of environmental factors on the expression of them before a generalized conclusion.

References
Table 1-Analysis of variances for tall fescue forage production traits in endophyte-infected and noninfected plant genotypes.

<table>
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<tr>
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<th>SDW</th>
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<td>**</td>
<td>**</td>
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**, NS Significant at the 0.01 level and Not Significant, respectively.

SFW: Shoot Fresh Weight, SDW: Shoot Dry Weight, RFW: Root Fresh Weight, RDW: Root Dry Weight, PHY: Plot Herbage Yield, TIL: Tiller numbers per hill and REG: Regrowth height.

Figure 1(a)- Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW) and plot herbage yield (PHY) of tall fescue clones as affected by endophyte infection. Values are means of six clones and three replications per clone. Means with the different letter are significantly different.
Figure 1(b)- Tiller number per hill and mean regrowth height of tall fescue clones as affected by endophyte infection. Values are means of six clones and three replications per each clone. Means with the different letter are significantly different. TIL: Tiller number and REG: Regrowth
Use of Nuclear Techniques in Fertigation Studies at NRCAM

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Abstract:
Recognizing the potential use of nuclear techniques in fertigation studies, the Nuclear Research Center for Agriculture and Medicine (NRCAM) implemented a series of experiments. The main objective was to improve water and fertilizer management in field and green house using nuclear and fertigation technibgies. The techniques included the use of $^{15}$N and $^{32}$P isotopes and neutron moisture gauge for N, P and water management respectively. Non-isotopic fertilizers applied through drip irrigation system and labeled fertilizer applied for isotopic subplots through bottles. The amount of water and fertilizers applied through the bottles was equivalent to the concentration applied through a single dripper. In the studies such parameters like evaluation of 1) nitrogen and phosphorus amounts derived from soil and fertilizer 2) the fertilizer use efficiency 3) water use efficiency and 4) yield, were taken into consideration.

For tomato fertigation the N- urea, P- phosphoric acid and water use efficiencies was found, %63, %23 and 16kg/m² respectively.
As a whole, results are indicative of significant role played by combined nuclear and fertigation technologies in developing more efficient fertilizer and water management strategies for crop production in arid and semi- arid zone.

Key words: crop yield, fertigation, fertilizer use efficiency, Isotope techniques, water use efficiency

Introduction
The only direct means of measuring nutrient uptake from the applied fertilizer is through the use of isotopes. Extensive work has been conducted using N- fertilizers labeled with the stable isotope $^{15}$N and P- fertilizers labeled with the radioactive isotope $^{32}$P. Initial work has been done with N and P utilizing isotopic methods, (Broeshart, 1978, Fried, 197, IAEA, 1970a, 1970b, 1971, 1974, 1975, 1978a, 1980, 1983a, FAO, 1980, Zapata and Hera, 1995).
In isotopic- aided fertilizer experiments, a labeled fertilizer is added to the soil and the amount of fertilizer nutrient that a plant has taken up is determined. In this way, different fertilizer practices (Placement, timing, sources, etc.) can be studied. The first parameter to be determined when studying the fertilizer uptake by a crop by means of the isotope techniques is the fraction of the nutrient in the plant derived from the (labeled) fertilizer (IAEA, 2001).

The nitrogen isotope composition, i.e. the $^{15}$N/ total N ratio, of any material can be expressed as atom % $^{15}$N or simply % $^{15}$N abundance. This isotopic ratio of a sample is measured directly in a single determination by optical emission or mass spectrometry. Phosphorus has one stable isotope ($^{31}$P) and several radioisotopes but only two of them ($^{32}$P and $^{33}$P) are suitable for agronomic studies. Detection efficiencies for both radioisotopes are high using modern liquid scintillation counters/ analyzers. The $^{32}$P can
be easily monitored because of its high beta energy and its use is limited to P uptake studies with duration of 60 up to 90 days due to its short half – life (IAEA, 2001). Research on soil water and irrigation often depends on determination of actual soil moisture at many different experimental sites, at different depths in the soil and with different irrigation and other treatments. Soil water measurement based on neutron scattering has been a valuable tool for the past 50 years because it possesses many of qualities such as simplicity, reliability, repeatability, cost effectiveness, and the method is non-destructive (IAEA, 2001). Recognizing the potential use of nuclear techniques in soil water and plant nutrition studies, the NRCAM implemented a series of experiments. The main objective was to improve water and fertilizer management in field and greenhouse, using nuclear and fertigation technologies.

Chemigation is the application of any chemical through irrigation water. This may include insecticides, fumigants, nematicides, fertilizers, soil amendments, and other compounds. By far, the most common form of chemigation is fertigation, which refers to fertilizer application in the irrigation water. Fertigation as an attractive technology in modern agriculture increases yield, fertilizer and water use efficiencies (Burt et al., 1995).

Materials and methods

The $^{15}$N and $^{32}$P methodologies are used for evaluation of N and P nutrients derived from urea and phosphoric acid fertilizers by plant. Neutron moisture gauge used for soil water measurements and irrigation program.

Recorded data

1- Dry matter yield (Dm) for plant parts.
2- Total N and P concentrations in dry matter.
3- Plant $^{15}$N abundance and $^{32}$P specific activity analyzed by emission spectrometry and LSC respectively.
4- Fertilizers $^{15}$N abundance and $^{32}$P specific activity.
5- The rates of N and P applications.

Calculations for experiment with $^{15}$N

1- $^{15}$N abundance -0.3663 = $^{15}$N excess
2- $^{15}$N excess in plant sample = $^{15}$N excess in fertilizer

3- Dry matter yield (kg/ha) = FW(kg) × $\frac{10000(m^2/ha)}{area\ harvested(m^2)} \times SDW(kg) \times SFW(kg)$

Where FW is fresh weight per area harvested and SDW and SFW are sub sample dry and fresh weights, respectively.

4- N yield (kg/ha) = DM yield (kg/ha) × $^{15}$N

5- Fertilizer N yield (kg/ha) = N yield(kg/ha) × $^{15}$N × $^{15}$Nff

6- %Fertilizer N utilization = $\frac{Fertilizer \ N \ yield}{Rate \ of \ N \ application} \times 100$
Calculations for experiment with $^{32}$P

$$\% \text{Pff} = \left( \frac{\text{Spesific activity of plant sample}}{\text{Spesific activity of labelled fertilizer}} \right) \times 100$$

Dry matter yield, P yield, fertilizer P yield and fertilizer P utilization percent calculated as for $^{15}$N equations.

Calculations for soil water measurement

Soil water storage in the plant rooting zone is calculated using water distribution profiles, which are assessed with neutron moisture gauge. The following equation is used to calculate the soil water storage.

$$S(t) = \sum \varphi \Delta Z$$

Where $S(t)$ is soil water storage, $\varphi$ is the volumetric soil water content and $\Delta Z$ is the depth of soil. The water balance approach was used to estimate crop water consumption. The difference between gain and losses of water over the depth $L$ (plant rooting zone) gives rise to a net change of soil water storage ($\Delta S$) which is measured with a neutron moisture gauge. The water balance equation is given as $I + P - (D + ET) - R = \pm \Delta S$ Where, $I$ is irrigation, $P$ is rainfall, $D$ is drainage, $ET$ is evapotranspiration and $R$ is run off.

Following experiments were conducted at the Nuclear Research for Agriculture and Medicine in Rajaie- Shahr, Karaj about 60 km west of Tehran. Located at altitude of 1310 m, latitude 36N, longitude 51E, with average 250 mm annual rainfall and 13.6ºc air temperature.

Experiment 1.

In year 1998 urea fertilizer and water use efficiency by tomato (Early Urbana VF) in sandy loam soil were investigated comparing trickle fertigation and conventional furrow irrigation – band fertilization. Following five treatments are replicated four times in a randomized complete block design.

1- Urea- N0= 0 mgNl⁻¹ trickle fertigation
2- Urea- N1=38 mgNl⁻¹ trickle fertigation
3- Urea- N2= 76 mgNl⁻¹ trickle fertigation (equivalent NS treatment)
4- Urea- N3=114 mgNl⁻¹ trickle fertigation
5- Urea- NS=500 kgNha⁻¹ conventional fertilization/ furrow irrigation

On the trickle irrigated plots, fertilizers were applied through the irrigation system by the use of two fertigators: one for the application of urea and the other one for the application of diammonium phosphate and potassium sulfate. Installed micro tubes in the system splitted the urea. Six drippers in the middle row of each plot were blocked and the plants received 2% $^{15}$Na.e. urea through bottles. The amount of water and fertilizers applied through the bottles was equivalent to the concentrations applied through a single dripper. Access tubes for neutron probe reading in all treatments were installed in two replications at the depth of 100 cm below the drippers in the middle of second row. Readings with the neutron gauge were taken before and after each irrigation at 30, 45, 60, 75 and 90 cm soil depth. Water consumption (ET) was calculated on the basis of the ratio of fruit – canopy dry matter weight to the unit of water consumed.

As it is shown on Table1, the highest total dry matter yield was found 11.4 tons/ ha for the N2 treatment. Where as, the NS treatment produced the lowest (6.5 ton/ha) dry matter yield. Highest value for total nitrogen use efficiency percent (N.U.E
% in comparison belonged to N1 treatment with 54%, which both parameters show statistical significance. The amount of irrigation water applied was 6450 and 12250 m$^3$/ha for trickle irrigation and conventional furrow irrigation treatments respectively. As it is shown in table 2. The highest water consumption was 99.4 cm for the NS treatment and the lowest water consumption was 52.9 cm for the N0 treatment. The water use efficiency was the highest for N1 treatment (94.4 kg/ha.cm). It was the lowest for NS treatment (33.2 kg/ha.cm).

Experiment 2.

The effect of fertigation intervals on nitrogen, phosphorus and water use efficiencies on tomato (Early Urbana VF) evaluated using $^{15}$N, $^{32}$P and neutron gauge in 1999-2000 at the N.R.C.A.M experimental field. The following five fertigation treatments were replicated four times in a randomized complete block design.

T1: fertigation in every irrigation.
T2: fertigation in every second irrigation.
T3: fertigation in every third irrigation.
T4: fertigation in every fourth irrigation.

The amounts of fertilizer used were in accordance with the soil chemical analysis. The experimental field received 344 and 250 kg N/ha in 1999 and 2000 as urea, 97 kg P/ha as phosphoric acid and 266 kg K/ha as potassium sulfate through the irrigation system by use of one fertigator. Four drippers in the middle row of each plot were blocked and the plants received 2% $^{15}$N a.e urea through plastic containers. In order to study phosphorus fertilizer use efficiency, 15 plants were separated by the fence and three drippers in the middle of each fence were blocked and plants received $^{32}$P labeled phosphorus through containers. Ortho phosphoric acid (4.5ml with activity of 17 mci) was made in the experimental reactor of Tehran. After dilution, the specific activity of the labeled fertilizer was 10.43 MBq/gm. The total amount of labeled fertilizer divided into 36 units and applied with each irrigation. The amount of water, fertilizer, fungicide and micronutrients applied through a single dripper. Determination of the activity (dps) of the plant materials was done by radio assay technique using appropriate detector (Beta Counter).

Access tubes (for neutron probe readings) were installed in duplicate to a depth of 100 cm below the drippers in the middle of the second row. Readings with neutron gauge were taken before and after each irrigation at 30, 60, 75 and 90 cm soil depth.

Table 3 shows the results of the experiment. The highest N.U.E was found with T1 treatment for 1999 and 2000. They were 42.0 and 62.8 respectively. The lowest N.U.E% was found with the T4 treatment being 35.3 and 47.8 for 1999 and 2000 respectively. The highest total dry matter yield was observed for T1 treatment for 1999 and 2000. They were 10.9 and 11.5 ton/ha (122 ton/ha fresh fruit yield) respectively. The lowest total dry matter yield was found with the T4 treatment being 8.7 and 8.8 ton/ha for 1999 and 2000 respectively. The results indicate that urea fertigation frequencies were not significant at %5 level related to dry matter, total nitrogen percentage and finally nitrogen use efficiency. This means that under general farm condition one can apply urea, phosphoric acid and potassium sulfate all together using one of the surveyed frequencies in this experiment.

Table 4 shows the results of phosphorus isotopic analysis in different parts of tomato. The highest amounts of dry matter yield were recorded for fruits (5.81 Ton/ha). In this respect, phosphorus fertilizer uses efficiency were %11.7, %7.8, %2.2 and %1.6 for fruit, leaf, stem and root respectively. 23.3 percent of phosphorus fertilizer transferred to plant organs and 76.7 percent remained in the soil.
Table 5 shows the fluctuation of phosphorus fertilizer use efficiency during six weeks of harvesting period. The highest amounts of dry matter yield and phosphorus yield were recorded during second and third weeks. Mutual effects of D.M.Y, total P and %PdfF were affected in the phosphorus use efficiency and consequently 65 percent of P was absorbed by the fruit during these two weeks. The amount of irrigation water applied was 8215 and 7596m3/ha in 1999-2000 respectively as it is shown in table 6 the highest fresh fruit yield were 116.5 and 121.9 ton/ha for T1 treatment in 1999-2000 respectively, while the lowest fresh fruit yield were 103.4 and 105.9 ton/ha for T4 treatment in 1999-2000 respectively. The highest water use efficiency was 14.2 and 16 kg/m3 for T1 treatment and the lowest (12.6 and 13.9 kg/m3) for T4 treatment in 1999-2000 respectively.

Conclusion

Two out of a series of experiments have been presented in this paper specifically on their methodologies. Application of nuclear techniques in evaluation of water and fertilizer use efficiencies in this research improved precision in interpretation of nutrients, water and plant relationships. Fertigation technology is recognized as a desirable system for crop production in arid and semi-arid regions and finally its potential use in our country requires more research.

References

### Tables

**Table 1.** Total dry matter and urea –N utilization of tomato 1998.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D.M.Y T.ha⁻¹</th>
<th>Total N %</th>
<th>N. yield Kg ha⁻¹</th>
<th>NdfF %</th>
<th>F.N.Y Kg ha⁻¹</th>
<th>N.U.E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>3.0 B</td>
<td>3.3</td>
<td>90</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N1</td>
<td>5.1 A</td>
<td>3.8</td>
<td>194</td>
<td>36</td>
<td>70</td>
<td>35 A</td>
</tr>
<tr>
<td>N2</td>
<td>5.2 A</td>
<td>3.7</td>
<td>192</td>
<td>35</td>
<td>67</td>
<td>22 B</td>
</tr>
<tr>
<td>N3</td>
<td>4.6 A B</td>
<td>3.5</td>
<td>161</td>
<td>39</td>
<td>63</td>
<td>16 B</td>
</tr>
<tr>
<td>NS</td>
<td>3.3 B</td>
<td>3.2</td>
<td>106</td>
<td>28</td>
<td>30</td>
<td>10 C</td>
</tr>
<tr>
<td>Canopy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>4.3 AB</td>
<td>2.2</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>5.2 AB</td>
<td>2.4</td>
<td>125</td>
<td>30</td>
<td>38</td>
<td>19 A</td>
</tr>
<tr>
<td>N2</td>
<td>6.2 A</td>
<td>3.2</td>
<td>143</td>
<td>30</td>
<td>51</td>
<td>17 A</td>
</tr>
<tr>
<td>N3</td>
<td>4.7 AB</td>
<td>2.7</td>
<td>127</td>
<td>48</td>
<td>61</td>
<td>15 A</td>
</tr>
<tr>
<td>NS</td>
<td>3.2 B</td>
<td>2.3</td>
<td>74</td>
<td>3B</td>
<td>22</td>
<td>7 B</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>N0</td>
<td>7.3 AB</td>
<td>2.6</td>
<td>185</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N1</td>
<td>10.3 A</td>
<td>3.1</td>
<td>319</td>
<td>34</td>
<td>108</td>
<td>54 A</td>
</tr>
<tr>
<td>N2</td>
<td>11.4 A</td>
<td>2.9</td>
<td>335</td>
<td>35</td>
<td>118</td>
<td>39 B</td>
</tr>
<tr>
<td>N3</td>
<td>9.3 B</td>
<td>3.1</td>
<td>288</td>
<td>43</td>
<td>124</td>
<td>31 B</td>
</tr>
<tr>
<td>NS</td>
<td>6.5 AB</td>
<td>2.8</td>
<td>180</td>
<td>29</td>
<td>52</td>
<td>17 C</td>
</tr>
</tbody>
</table>

- Values in columns followed by the same letter are not significantly different at the 5% probability level.

**Table 2.** Effect of irrigation method and N rates on fruit dry matter yield, evapotranspiration (ET) and water use efficiency (WUE) according to neutron probe calculation for tomato in 1998.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry matter (ton/ha)</th>
<th>ET(cm)</th>
<th>WUE(kg/ha.cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>3</td>
<td>52.9</td>
<td>56.7</td>
</tr>
<tr>
<td>N1</td>
<td>5.1</td>
<td>54</td>
<td>94.4</td>
</tr>
<tr>
<td>N2</td>
<td>5.2</td>
<td>59.4</td>
<td>87.5</td>
</tr>
<tr>
<td>N3</td>
<td>4.6</td>
<td>61.8</td>
<td>74.4</td>
</tr>
<tr>
<td>NS</td>
<td>3.2</td>
<td>99.4</td>
<td>33.2</td>
</tr>
</tbody>
</table>

**Table 3:** Dry matter yield, total N, %NdfF, fertilizer N yield and %N use efficiency of tomato (Total) as affected by different intervals urea application during (1999-2000).

<table>
<thead>
<tr>
<th>Trea.</th>
<th>D.M.Y ton/ha</th>
<th>Total N %</th>
<th>NdfF %</th>
<th>F.N.Y kg/ha</th>
<th>N.U.E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>10.9</td>
<td>11.5</td>
<td>2.4</td>
<td>2.4</td>
<td>55.6</td>
</tr>
<tr>
<td>T2</td>
<td>9.4</td>
<td>9.8</td>
<td>2.4</td>
<td>2.4</td>
<td>57.0</td>
</tr>
<tr>
<td>T3</td>
<td>9.2</td>
<td>9.5</td>
<td>2.4</td>
<td>2.4</td>
<td>56.9</td>
</tr>
<tr>
<td>T4</td>
<td>8.7</td>
<td>8.8</td>
<td>2.7</td>
<td>2.6</td>
<td>52.7</td>
</tr>
<tr>
<td>%CV</td>
<td>11.2</td>
<td>17.7</td>
<td>6.3</td>
<td>7.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>

- Values in columns followed by the same letter are not significantly different at the 5% probability level.
Table 4. Dry mater yield, Total phosphorus, P yield, Phosphorus derived from fertilizer (PdfF), Fertilizer Phosphorus yield (F.P.Y), Phosphorus fertilizer use efficiency (P.U.E), in different parts of tomato 2000.

<table>
<thead>
<tr>
<th>Plant parts</th>
<th>D.M.Y Ton/ha</th>
<th>Total P %</th>
<th>P yield Kg/ha</th>
<th>PdfF %</th>
<th>F.P.Y Kg/ha</th>
<th>P.U.E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>3.3 B</td>
<td>0.57 A</td>
<td>18.92 B</td>
<td>39.8 ns</td>
<td>7.53</td>
<td>7.76 A</td>
</tr>
<tr>
<td>Root</td>
<td>0.87 C</td>
<td>0.40 B</td>
<td>3.52 C</td>
<td>44.1 ns</td>
<td>1.55</td>
<td>1.60 B</td>
</tr>
<tr>
<td>Stem</td>
<td>1.83 C</td>
<td>0.28 C</td>
<td>5.04 C</td>
<td>42.2 ns</td>
<td>2.13</td>
<td>2.20 B</td>
</tr>
<tr>
<td>Fruit</td>
<td>5.81 A</td>
<td>0.54 A</td>
<td>31.59 A</td>
<td>35.9 ns</td>
<td>11.34</td>
<td>11.69 A</td>
</tr>
<tr>
<td>%CV</td>
<td>21</td>
<td>9</td>
<td>24</td>
<td>32</td>
<td></td>
<td>33</td>
</tr>
</tbody>
</table>

- Data: mean of four replications
- The same letter in a column refers to no significant difference in 1% level (Duncan's test)

Table 5. The comparison of fertilizer phosphorus use efficiency during six harvesting times of tomato fruit 2000.

<table>
<thead>
<tr>
<th>Harvesting time (Days after cultivation)</th>
<th>D.M.Y Ton/ha</th>
<th>Total P %</th>
<th>P yield Kg/ha</th>
<th>PdfF %</th>
<th>F.P.Y Kg/ha</th>
<th>P.U.E %</th>
</tr>
</thead>
<tbody>
<tr>
<td>83</td>
<td>0.58 C</td>
<td>0.52 B</td>
<td>3.00 B</td>
<td>33.66 AB</td>
<td>1.01</td>
<td>1.04 B</td>
</tr>
<tr>
<td>90</td>
<td>2.16 A</td>
<td>0.43 B</td>
<td>9.31 A</td>
<td>45.96 A</td>
<td>4.28</td>
<td>4.41 A</td>
</tr>
<tr>
<td>99</td>
<td>1.58 B</td>
<td>0.57 B</td>
<td>9.03 A</td>
<td>34.65 AB</td>
<td>3.13</td>
<td>3.23 A</td>
</tr>
<tr>
<td>104</td>
<td>0.52 C</td>
<td>0.47 B</td>
<td>2.44 B</td>
<td>45.53 A</td>
<td>1.11</td>
<td>1.14 B</td>
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<tr>
<td>111</td>
<td>0.71 C</td>
<td>0.80 A</td>
<td>5.69 AB</td>
<td>21.98 B</td>
<td>1.25</td>
<td>1.29 B</td>
</tr>
<tr>
<td>124</td>
<td>0.26 C</td>
<td>0.81 A</td>
<td>2.12 B</td>
<td>25.99 B</td>
<td>0.55</td>
<td>0.57 B</td>
</tr>
<tr>
<td>%CV</td>
<td>24</td>
<td>12</td>
<td>33</td>
<td>23</td>
<td></td>
<td>29</td>
</tr>
</tbody>
</table>

- Data: mean of four replications
- The same letter in a column refers to no significant difference in 1% level (Duncan's test)

Table 6: Effect of different fertilization intervals on fresh fruit and water use efficiency (WUE) for tomato 1999-2000.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh 1999</th>
<th>Fruit (ton/ha) 2000</th>
<th>Water use 1999</th>
<th>Efficiency (Kg/m³) 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>116.5 A</td>
<td>121.9</td>
<td>14.2</td>
<td>16</td>
</tr>
<tr>
<td>T2</td>
<td>108.3 A</td>
<td>112.5</td>
<td>13.2</td>
<td>14.8</td>
</tr>
<tr>
<td>T3</td>
<td>108.5 A</td>
<td>111.8</td>
<td>13.2</td>
<td>14.7</td>
</tr>
<tr>
<td>T4</td>
<td>103.4 A</td>
<td>105.9</td>
<td>12.6</td>
<td>13.9</td>
</tr>
</tbody>
</table>

- Values in columns followed by the same letter are not significantly different at the 5% probability level.
A novel strategy for modeling, synthesis and plant expression prediction of the Bacillus thuringiensis cry genes

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Abstract:

A Bacillus thuringiensis cry3a δ-endotoxin gene was designed for optimal expression in plants. The modified cry gene has the codon usage pattern of average dicot genes and does not contain AT-rich nucleotide sequences typical of native Bt cry genes. As a result of the modification the AT content was reduced from 64% to 51% and all known DNA sequences that might contribute to RNA instability in plants were eliminated. We assembled the 1.8 kb cry3a gene in 4 blocks and every block assembled from many complementary oligonucleotides. A new strategy was used for the chemical synthesis of the modified gene. The oligonucleotides 50-55 (for the first strand) and 30-35 (for the second strand) bp long were synthesized by means of phosphoramidite chemistry. The combined mixture of the first and second strand oligonucleotides was exposed to DNA polymerase I and the dNTPs to complete synthesis of the second strand, and then T4 DNA ligase was added to join the adjacent oligonucleotides. The assembled fragments were subjected to PCR, and then cloned to T-vectors. To obtain the complete gene sequence, all the four fragments in the correct frame were cloned into a pGEM vector. A comparative analysis of expression in E.coli cells has shown that the synthetic gene was 1.5 times less active than the native gene. Quantitative and qualitative assays of lichenase in the hybrid proteins and of gene expression has shown that the synthetic gene was expressed approximately 10-fold greater than the native gene. These results suggest that a high level of expression of the synthetic cry3a gene can be expected in plant cells.

Keywords: Bacillus thuringiensis var. tenebrionis, δ-endotoxins, Colorado potato beetle, cry3a, synthetic gene, yeasts.

Introduction:

In the post genomic era, thousands of unknown proteins have become available for study. While in theory, the structures and functions of many of these proteins may be determined by comparative analysis, in most cases overexpression and purification of target protein will be necessary. Although the use of naturally occurring genes might appear to be quickest approach, many such genes will prove to be suboptimal for cloning and overexpression in heterologous systems like E.coli, yeasts and plants. The potential problems include high A-T contents, codon bias and complex intron/ exons structures. For example, Bacillus thuringiensis cry genes have many splicing and polyadenylation sites, the ATTTA sequences, mRNA degradation signals and transcription termination sites as well as the codons that are rare in plants (Dennis et al. 1992). Efforts for enhance the expression of these genes include expressing only the region of the gene encoding the insecticidal domain, modifying the 59 and 39 UTRs, generating protein fusions, and using a variety of strong promoters (Martinez. et al. 2002). The mRNA and protein levels were eventually increased by resynthesizing the genes to be more “plant like.” Increasing the G-C content of the genes may eliminate potential RNA-processing signals. The problem can occur
at one or more steps in gene expression. mRNA accumulation may be limited at the level of transcription by sequences within the coding region that adversely affect transcription initiation or elongation. Alternatively, the problem may occur post-transcriptionally (Crickmore et al. 1998, Kuo et al. 1996) as a result of aberrant splicing and/or degradation of the transcript. An approach to overcoming the complications in cloning is gene synthesis. In this approach, the protein coding sequence can be directly optimized for the expression system of choice. Variants of this strategy include oligonucleotide ligation, the fokI method and self-priming PCR. A particularly appealing method, due to its inherent simplicity, is assembly PCR. This involve generating overlapping oligonucleotides which, when assembled form the template for the gene of interest. The oligonucleotides are then repetitively extended by PCR, to assemble one fragment or the full-length gene in a single step (David and Lubowski, 2002).

In this work, using molecular biology software (DNAWorks, DNAsis, Clone and Vector Xpression), we redesigned cry3a gene sequence to enhance high expression in potato cells for control of Colorado potato beetle (Leptinotarsa decemlineata). The three-dimensional structure of the Cry3a toxin consists of three functional domains: (I) a cluster of seven \(\alpha\)-helices predicted to be involved in membrane interaction; (II) three antiparallel \(\beta\)-sheets involved in receptor binding; and (III) a \(\beta\)-sandwich implicated in receptor binding and ion channel activity (Perlak & Stone, 1993). A new strategy was used for the chemical synthesis of the modified gene. A set of oligonucleotide sequences (composing the gene of interest) that have been optimized to match the codon bias of the potato for expression and highly homogeneous melting temperatures of all overlapping oligonucleotide sections were created. Then, these oligonucleotides were combined and assembled in a one-step PCR protocol to form the synthetic gene. The native and synthetic genes were then fused with the reporter gene licB which codes for a thermostable lichenase (Pirusian et al. 1998 & 2002) and cloned into bacterial and yeast expression vectors. Using the quantitative and qualitative assays of lichenase in the hybrid proteins and of gene expression was assessed expression quantity of wild type and synthetic genes in E.coli and yeasts cells.

**Material and Methods**

**Molecular cloning of wild type cry3a gene:** Standard protocols were used for PCR and vector construction (Sambrook et al. 1989). The 1.8 cry3a gene was isolated from the genomic DNA of Bt var. tenebrionis by PCR using primers: 5’-ggatccatgcagataataatacg-3’ and 5’-gagctcattcactggaataaattcaattt-3’. The amplified fragment then was isolated and purified from agarose gel after electrophoresis. Isolated fragment was cloned to pUC18, and then into pET-32a(+) and yeast expression vector pGAL. The resulting plasmids pET-cry3a and pGal-cry3a were obtained.

**Oligonucleotide synthesis and phosphorylation:** Synthetic gene was designed and synthesized in four fragments with length about 500 bp. Besides, in the beginning and end of each fragments were added restriction sites, which exist inside the sequence of native cry3a gene (Ndel, Bsu36I, XbaI). Oligonucleotides were prepared by the solid phase phosphoramidite triester couplings approach, using ASM-700 and ASM-800 (OOO "Bioset", Novosibirsk, Russia) DNA synthesizer. The oligonucleotide is synthesized in 3’ to 5’ direction while attached covalently to a solid support (solid phase synthesis). The building blocks used for synthesis are DNA phosphoramidite nucleosides that are modified with different protection groups. In Figure 1 the DNA synthesis cycle is outlined schematically. Synthesis starts with de-blocking (A) i.e. the cleavage of the 5’ protection group dimethoxytrityl (DMT). An activated phosphoramidite nucleoside couples with the free 5’hydroxyl function (B). Typically coupling efficiency is ca.
99%. Since the coupling efficiency is not 100%, a small percentage of truncated sequences are produced at every coupling step. If these failure sequences are allowed to further react, unwanted deletion mutants would result. This problem is overcome largely by capping the remaining free 5’ hydroxyls through acetylation (C). Some molecules fail to cap and continue to participate in additional synthesis cycles, resulting in near full-length molecules that contain internal deletions, the so-called (n-x)mer species. After coupling, the DNA bases are connected by an unstable phosphite triester. It is converted to a stable phosphotriester linkage by oxidation (D). The oxidation step completes one cycle of oligo synthesis. DNA synthesis was continued with the removal of the DMT group at the 5’-end of the growing chain, starting another cycle of nucleotide addition. Synthesis cleavage from support and deprotection was achieved by treatment with concentrated ammonium hydroxide.

Crude oligonucleotide mixtures were purified using an oligonucleotide purification cartridge as described by McBride et al. 1988. Phosphorylation of oligonucleotides was performed enzymatically using bacteriophage T4 polynucleotide kinase. A kinasing reaction typically consisted of 2 µg of oligonucleotide in linker kinase buffer, plus 1 mM ATP and 20 units of T4 polynucleotide kinase in a total volume of 50 µl. Prior to addition of ATP and enzyme, the reactions were heated to 100 °C for 5 min, and then placed immediately on ice. ATP and enzyme were then added and incubated for 1 h at 37 °C.

Ligation of oligonucleotide segments: Each oligonucleotide segment (first and second strands) of the synthetic gene was enzymatically phosphorylated as described, with the exception of two 5’-terminal oligonucleotides which were left unphosphorylated in order to prevent the formation of concatemers. However, they were incubated in the same volume of linker kinase buffer for the same length of time as oligonucleotides undergoing phosphorylation. All oligonucleotides were then heated separately to 95 °C for 5 min, complementary pairs were mixed together at 95°C for another 5 min, and annealing was allowed to occur by slow cooling to room temperature. Annealed pairs forming one block were mixed together for ligation. The combined mixture was exposed to DNA polymerase I and the dNTPs to complete synthesis of the second strand. Ligation were performed with 1mM ATP and 1 to 3.5 units of T4 ligase at 15 °C for 2 h.

Purification and PCR of ligated blocks: Each ligated block mixture was subjected to electrophoresis through agarose gel according to Zupancic et al, 1988. The band corresponding to the correctly assembled segment was purified from the gel by phenol extraction, ethanol precipitated and then used for PCR amplification. PCR was used to amplify the correct sized fragment. After electrophoresis of the ligation reaction, the region of the gel where the correctly ligated block was expected to migrate was excised with a razor blade, even if no DNA were visible. An agarose plug was removed from the center of the gel slice and used as the template for PCR with the 5’ end oligonucleotides from each strand used as primer at a concentration of 0.3 mM. Because of the large size of the primers, annealing and extension were performed at the same temperature of 72°C. thirty cycles of denaturation and extension/annealing were performed.

Cloning and sequencing of amplified blocks: The assembled fragments were then cloned, either directly or by digesting with the appropriate restriction enzymes, to check the nucleotide sequence. The ligation products were transformed into XL1blue E.coli cells and selected for on LB plates with 50 µg/ml ampicillin. The plasmids isolated were screened by either restriction digest analysis or PCR using primers complementary to vector sequences flanking the synthetic gene, and plasmids containing the fragment of interest were sequenced in both the forward and reverse directions.
Production of Cry3a protein in E.coli: For obtaining plasmid pGEM-f1-f2-f3-f4 (full synthetic gene), were conducted some intermediate cloning stages. The native and synthetic genes were then fused with the reporter gene licB which codes for a Clostridium thermocellum thermostable lichenase (licB) (Piruzian et al. 1998, 2002), and were cloned into bacterial expression vector pET32(a) behind the T7 promoter and received pET-cry3a-licB and pET-synthetic-licB constructions. Then these constructions were transformed to E.coli (strain BL21). E.coli clones were grown for Cry3a protein production in LB in the presence of antibiotics. Expression was induced in log-phase cultures (absorbance at 600 nm 0.7) with 1mM isopropyl thiogalactoside (IPTG) and cells were harvested after overnight incubation. Cells were pelleted, washed in 5X TE (1X TE is 10 mM Tris-HCl pH 7.5, 1mM EDTA), resuspended in the same buffer and disrupted by sonication or in a 8 M urea solution. Cry protein, along with cell debris, were concentrated from the crude E.coli lysate with a 12000 g centrifugation.

Production of Cry3a protein in yeasts cells: The natural and synthetic cry3a coding regions, contained within 1.8 kb were cloned to yeast expression vector pGal behind the Gal promoter (galactose inducible promoter) and then were fused with reporter gene licB. pGal-cry3a-licB, pGal-synthetic-licB were introduced into YPH 857 yeast cells using the lithium-acetate protocol (Soni et al. 1993). Induction of the Gal promoter was achieved by growing the S. cerevisiae transformants on agar plates or in liquid medium containing galactose as a carbon source. Yeast cells were pelleted and washed with water. To the cell pellet an equal volume of glass beads (425-600 µm in diameter; Sigma) and 200-400 µl of TRIS-HCl buffer (pH 8.0) was added. The cells were then disrupted for 90 s in a Disintegrator (B. Braun Melsungen, Germany). The resulting cell extracts were clarified by centrifugation at 12,000 g for 10 min.

Protein determination and enzyme assay: Lichenase activity was measured by a plate test according to Teather and Wood, 1982. Bacterial or yeast colonies were seeded onto plates of the appropriate medium. The colonies that appeared were covered with agarose containing 0.1% lichenan. The plates were then incubated for 1-5 h at 65°C, stained for 15 min with 0.5% Congo Red solution, and washed with 1 M NaCl until transparent spots of the hydrolyzed substrate became apparent. Protein was measured according to Bradford, 1979, using BSA as standard. Lichenase activity in the lysates was determined as described previously at 65°C using lichenan as substrate (Piruzian et al. 1998, 2002). Reducing sugars released from the substrate were determined with the dinitrosalicylic (DNS) reagent according to Wood and Bhat, 1988. The reaction mixture contained 200 µl of 0.5% lichenan and 100 µl of the protein sample. It was incubated for 10-20 min, then 1.2 ml of the dinitrosalicylic reagent was added, and the mixture was heated at 100°C for 15 min. The concentration of colored product was determined with an Ultraspec II spectrometer (LKB). Glucose standards as well as enzyme and substrate blanks were also assayed. One unit of lichenase activity is defined here as the amount of enzyme that produces 1 µmol of reducing sugars (as glucose equivalent) per min per mg of protein. All the activity values are averages of at least three independent assays performed in duplicate. The enzyme was characterized by the zymogram method described earlier (Piruzian et al. 1998). The bands of active enzyme appear as clear spots since the dye binds only to unhydrolyzed lichenan.

Results and Discussion

Design of the cry3a coding sequence: The purpose of redesigning the Bt var.tenebrionis cry3a gene was to create a synthetic gene that would be highly expressed in plant cells and thus confer resistance to CPB larvae in transgenic potatoes. The cry3a gene has an open reading frame equivalent to a 73 kDa protein (Secar et al., 1987). To make the cry3a gene more convenient for
plant, we referred to the codon usage table of many dicot and monocot genes compiled by Murray et al. 1989, 1991. In the synthetic gene, the cry3a sequence was modified to (1) contain codons preferred by plant genes, (2) avoid CG and TA dinucleotides at codon positions 2 and 3, (3) attain an A-T nucleotide base composition similar to that found in plants, (4) eliminate sequences that might cause mRNA destabilization and splicing, (5) minimize secondary structures hairpins, and (6) optimize the ATG flanking nucleotides for protein translation initiation. The redesigned gene differs from the native gene in 21% of its nucleotides.

**Synthesis of modified cry3a gene sequence:** A new strategy was used for the chemical synthesis of the modified gene. First the gene sequence was divided into four fragments about 500 bp long. Then, the coding (first) strand of each of the fragments was subdivided into 50-55 bp fragments, and the corresponding oligonucleotides were synthesized by means of phosphoramidite chemistry. Oligonucleotides were prepared by the solid phase phosphoramidite trimester couplings approach, using a synthesizer. This was followed by synthesizing a set of supporting complementary oligonucleotides 30-35 bases long which were complementary to the junctions between first chain oligonucleotides, so that 15-17 5'-end bases of the supporting oligonucleotides were complementary to the corresponding 3'-end of one oligonucleotide of the first strand and the remaining 15-17 3'-end bases of the same supporting oligonucleotide were complementary to the corresponding bases of the next first strand oligonucleotide. The mixture of first strand oligonucleotides (50-55 bases long) was phosphorylated. After inactivation of the enzyme, a mixture of the supporting oligonucleotides (30-35 bases long) at the same concentration was added. The combined mixture was exposed to DNA polymerase I and the dNTPs to complete synthesis of the second strand, and then T4 DNA ligase was added to join the adjacent oligonucleotides. The assembled fragments were then subjected to PCR, the product was gel purified and cloned, either directly or by digesting with the appropriate restriction enzymes, to check the nucleotide sequence (fig 2).

**Cloning of assembled fragments for complete gene construction:** The complete gene sequence was achieved by cloning of all the four fragments in the correct reading frame to a pGEM vector. For obtaining plasmid pGEM-f1-f2-f3-f4cry3a (synthetic) conducted some intermediate cloning stages. pGEM-f1-f2 was obtained by cloning of the Ndel-fragment of pGEM-f2, (containing the second fragment of the synthetic gene) to pGEM-f1, hydrolyzed on site Ndel. For obtaining pTZ-f1-f2-f3, Xbal-BamHI fragment (f3) from pQE-81-f3 cloned to pTZ-LicB, hydrolyzed by Xbal and BamHI endonucleases, with removing the licB sequence. After that, Bsu36I-Apal fragment (f1-f2), was cloned to plasmid pTZ-f3, hydrolyzed by endonucleases Bsu36I and Apal, and obtained plasmid pTZ-f1-f2-f3. Plasmid pGEM-f1-f2-f3-f4cry3a (synthetic) was obtained by cloning of Xbal-PstI fragment (f1-f2-f3) to pGEM-f4, hydrolyzed by Xbal and PstI endonucleases (fig 3).Such a strategy ensured the correct positioning of all oligonucleotides of the first strand, and hence the correct assembly of the target fragment and the whole gene.

**Use of a new reporter system for prokaryotic and eukaryotic cells based on the thermostable lichenase from Clostridium thermocellum:** In this work we used the lichenase reporter system. Lichenase is a thermostable enzyme that specifically hydrolyzes β-1,4 linkages adjacent to β-1,3 linkages in mixed-linkage β-glucans, but does not attack pure 1,3 or 1,4 linkages. Lichenase activity is not present in most bacteria, yeast, mammals, or plants, which make the enzyme a good reporter for these heterologous systems. It was shown that the thermostability of lichenase allows one easily to test for its expression against the background of thermolabile bacterial, yeast and plant enzymes. Zymogram analysis of the expression of the lichenase gene reveals the enzyme activity not only at a size corresponding to the predicted molecular mass of the enzyme,
but also in a band of lower molecular mass. The high activity and thermostability of the \textit{C. thermocellum} lichenase made it a good candidate for use as a reporter protein in bacterial, yeast, and mammalian cells. The high specific activity of the enzyme (2000 U) makes possible qualitative and quantitative measurements even at low levels of expression. Based on the results of the plate tests, the lichenase gene was used as a selective marker for screening of bacterial and yeast transformants. This kind of selection is very convenient for fast screening of bacterial and yeast transformants expressing the \textit{licB} gene under the control of both constitutive and inducible promoters. No background activities or changes in the growth of the transformed cells were observed in the model systems used. The zymogram method can be used instead of Western blotting for estimation of the expression level and size of fusion proteins (fig 4).

\textit{Inducible expression of the (native and synthetic) cry3a genes in E.coli:} To explore the expression level of both hybrid cry3a genes in \textit{E.coli} cells, BL21 strain of \textit{E.coli} was transformed with pET-cry3a-licB and pET-synthetic-licB. For this purpose, were used many quantitative (measuring of lichenase activity and protein concentration), and qualitative (zymogram, electrophoregram, Petri dish test, Congo Red test and DNS test) assays. The qualitative detection of lichenase activity by the zymogram and electrophoregram methods allowed one to determine directly the molecular masses of lichenase and fused Cry3a proteins. The molecular masses of lichenase and fusion Cry3a proteins measured in this work were in good agreement with theoretically predicted masses (100 KD) (Fig 4). A comparative analysis of expression in \textit{E.coli} cells has shown that the synthetic gene was 1.5 times less active than the native gene, which may reflect the fact that the codon composition of the synthetic gene was optimal for eukaryotes.

\textit{Inducible expression of cry3a genes in yeast cells:} To analysis of cry3a genes (native and synthetic) expression in eukaryotic cells and ascertain the feasibility of using lichenase gene as a reporter gene in yeast, we constructed yeast expression vectors in which the synthetic and native cry3a genes with licB sequence were placed under the control of an inducible (Gal) promoter. Yeast cells were transformed with the recombinant plasmids pGAL-cry3a-licB, pGal-synthetic-licB, as well as with positive (pGal-licB) and negative (pGal) controls. Transformants were streaked onto medium containing galactose, grown for 16 h, and lichenase activity was visualized by a plate test and zymogram (fig 4:C,D). All the cry3a-licB, synthetic-licB and licB transformants obtained expressed lichenase activity on medium with galactose. Transparent spots due to lichenase activity were observed around the yeast cells harboring all three constructions. The quantitative and qualitative assays of lichenase in the hybrid proteins and of gene expression in yeast cells has shown that the synthetic gene was expressed approximately 10-fold greater than the native gene. These results suggest that a high level of expression of the synthetic cry3a gene can be expected in plant cells.

\textbf{References}


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Figure legends:

Figure 1: Solid-phase oligonucleotide synthesis cycle (phosphoramidite chemistry).
Figure 2: The Scheme of assembly of a synthetic gene fragment.
Figure 3. The cloning and ligation scheme of different synthetic fragments of cry3a.

Figure 4. A. Zymogram of bacterial protein extracts at the presence of 0.1% lichenin as a substrate. 1- markers, 2- cry-licB, 3- synthetic-licB, 4- licB. B. Electrophoregram of bacterial protein extracts, 1- cry-licB, 2- synthetic-licB, 3- BL21 extracts. C. Zymogram of yeast protein extracts at the presence of 0.1% lichenan as a substrate, 1- pGAL-licB, 2- pGAL-synthetic-licB, 3- pGAL-cry-licB, 4- marker. D. Electrophoregram of yeast protein extracts: 1-marker, 2- pGAL-licB, 3- pGAL-synthetic-licB, 4- pGAL-cry-licB.
Characterization of Wheat Ribosomal RPL3 Gene with Respect to Fusarium Head Blight

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Abstract
Fusarium ear rot of maize and fusarium head blight of wheat and barley are serious diseases, limiting production of corn and small grains in many parts of the world. Severe yield reduction and contamination of fusarium mycotoxin of grains cause large economic losses. The most important causal agent of the disease is Fusarium graminearum (Gibberella zeae), which produces predominantly the trichothecene toxin deoxynivalenol (DON) and its acetylated derivatives. Trichothecenes inhibit eukaryotic protein synthesis, the proposed role of due fungal toxins is to block or delay the expression of defense related proteins induced by plants.

One approach to improving trichothecene tolerance and fusarium resistance is to prevent the binding of DON to ribosomal protein L3 (RPL3) in the cell by modifying the gene structure and reducing DON affinity for binding to protein RPL3 are responsible for yeast resistance to DON. There are a few reports suggesting that modified RPL3 confers improved resistance of transgenic plants to DON.

An important question is whether naturally existing alleles of RPL3, which confer increase DON resistance, can be found in plant genetic resources and exploited for wheat breeding.

The goal of our work was to characterize the RPL3 gene family of wheat and comprising resistance to sensitive cultivars and stabilize some molecular marker in this gene. The available sequences will provide more in-depth knowledge and understanding in designing an approach to engineer high-level constitutive resistance, which is not easily overcome by pathogen.

Key Words: Ribosomal protein L3, fusarium head blight, Deoxynivalenol

Introduction
Gibberella ear and stalk (maize) and fusarium head blight or scab (wheat, barely and oat) are globally important corps diseases caused by Fusarium graminearum. Fusarium head blight (FHB) causes yield losses and severe quality losses mainly by accumulation mycotoxin in the grains (3). Production of mycotoxin not only contributes to virulence of Fusarium graminearum but also causes some symptoms in human and animals (14, 7). One of the major and most important fusarium mycotoxin is deoxynivalenol (DON), a member of trichothecens. DON mycotoxin inhibits elongation or/and termination of translation (4, 15, 2), so interferes with the expression of defense related proteins.

Correlation evidence is available that toxin resistance significantly contributes to field resistance of wheat (1). A few cultivars have been shown to be resistant to fusarium head blight. These include the Chinese line Su Mei 3 and Brazilian line Frontana. Frontana is not only highly resistant to FHB but also highly tolerant of DON (9). Except some mutants yeast (9,5) no eukaryotic organism has been shown to display such resistance to trichothecens.

In Saccharomyces cervisiae, tolerance to trichothecene trichodermin was found to be conferred by a single gene known as tcm1. The wild type of tcm1 gene codes ribosomal protein L3 (6). Further more, there are some other mutants in tcm1 conferring DON resistance in yeast (Adam, G .personal communication). Evidently, this category of mutation can occur in wheat.
So far, there is no report of any sequence information regarding wheat RPL3 gene. At this point, the need of an hour is characterize the RPL3 gene in wheat cultivars. In the present work, we have characterize and the RPL3 gene from resistant and sensitive cultivar of wheat.

**Materials and Methods**

Wheat ESTs data were screened in gene bank and primers were design based on the available sequences and tomato and yeast RPL3 sequences. All primers were synthesized by MWG AG Company. Four cultivars of wheat, two resistant to fusarium head blight (Frontana and LI) and two sensitive to fusarium head blight (Falat and Remus) were studied. Total mRNA was extracted from leaves and cDNA was synthesis by using oligo dT primer. cDNA was amplified by specific primers. PCR products were cloned in pBlues KS vector and sequenced. MWG Company did sequencing. (Germany)

The sequences were studied using DNAStar software. Pair-wise alignments of sequences were determined using the Wisconsin GCG software package.

**Results**

Full length RPL3 cDNA has 1170 bp length and it codes a protein with 389 amino acids. There are two groups of RPL3 homeologs, As (A1, A2, and A3) and Bs (B1, B2, B3) in wheat. Based on amino acid alignment, there is a high level of homology in side A and inside B groups and in a lower level between A and B. (Figure 1).

Comparing of RPL3 proteins in resistant and sensitive cultivars, did not display any difference and all six homeologs shows identity in all cultivars. Figure 2 shows alignment of one of the homeologs in four cultivars.

The RPL3 cDNA sequences were submitted in the gene bank with accessions number AY343327-AY343330 and AY347531-AY347533

**Discussion**

cDNA from two resistant cultivars and two susceptible cultivars were sequenced and it was shown that wheat express all of the six homeologs of RPL3. There are some differences amount six homeologs of RPL3 in wheat, but no difference were observed amount cultivars.

three major DON resistance mechanisms were identified in yeast: first reduce toxin due to ABC transporter protein PDR5p (molecular efflux pump), second detoxification by modifying enzymes and, third modification of ribosomal target by amino acid change in ribosomal protein L3 (RPL3p) (11). It was reported that resistant cultivars of wheat contained low concentration of DON, whereas susceptible cultivars of wheat contained relatively high concentration (8).

In addition, detoxification of fusarium mycotoxin by variant enzymes such as UDP-glycosyl transferase (13) and Glutathione S-transferase (12) is reported in some plants. In suspension culture of the resistance wheat cultivar, Frontana, active degradation of DON was reported(10).

According to our data, it seems that nature does not select the third mechanism for fusarium resistance in wheat. On the other hand, there are some reports that showing that a modified versions of the RPL3 protein confer tolerance of DON to transgenic plants (6, 7), but utilization of this modified RPL3 is toxin dependent. It means that modified RPL3 is unstable or could be less competitive during ribosome assembly and it is not suitable for selection.

The molecular resistance mechanisms against FHB in wheat are complex and if there is any involvement of RPL3 protein in resistance mechanism, it should be happened at posttranslational steps.
References
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Figure 1: Identity present between six RPL3 homeologs in wheat.

Figure 2: Alignment of A2 homeologs in five cultivars of wheat.
Crown rot disease on wheat caused by the new species, Fusarium Pseudograminearum in the northwest of Iran.

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Abstract
The crown rot disease of wheat was studied during in four provinces including Qazvin, Zanjan, east Azarbyjan and Ardabil in northwest of Iran. Different wheat fields in studied areas were visited and samples of the suspected plants showing symptoms such as withering, whiting of spikes, growth reduction and white heads collected and transferred to laboratory. Samples were cultured in media and isolated fungi belongings to five genera were identified. Generally, the most frequently isolated pathogen was Fusarium pseudograminearum as a soil borne fungus. This species normally caused crown rot resulting in severe damage in several locations under dry conditions in spring season. The disease caused losses from 18 - 45.5% in the fields where the season and crop rotation allowed the disease to build up. Environmental conditions and genetic susceptibility of cultivars were the two main factors affecting the incidence of diseases.

Keywords: Wheat, Crown rot, Fusarium pseudograminearum, Iran

Introduction
Wheat (Triticum aestivum L.) covers about 30% of the world’s cultivated land and is the most important agricultural commodity in international trade (karimi 1992). The most important agricultural crop of Iran is wheat and is cultivated approximately in 4.5 million hectares. Its production was 8.7 million tons in 1999. Crown rot is a significant disease of small grain cereals, caused mostly by Fusarium species and is the main disease limiting yield of wheat in northwest Iran (Froutan et al. 1995).

Since the plant is adapted to different climatic areas from cool to warm conditions, many soil borne fungi can cause seedling blight and common root rot on wheat and result in yield losses (Wise, 1998). However, crown rot of wheat in Iran is caused mostly by the new species named Fusarium pseudograminearum (Aoki & Donnell, 1999) formerly known as F. graminarum (Group 1). Generally the increased incidence and economic importance of the disease has been linked to environmental conditions such as dry weather and use of susceptible varieties.

Generally, crown rot is the most common diseases of wheat in northwest Iran and usually appears after flowering when white heads can be seen scattered in the crop. It usually occurs in large patches and is more common on the lower sides of paddocks. The disease acquires economic proportions in the northwest of Iran, especially in East Azarbyjan, Ardabil and Zanjan provinces. It is also a serious problem in Australia (Burgess and Patterson, 1996) and USA (Smiley et al. 1996). In addition the disease has been reported from the wheat tracts of South Africa (Klaasen et al. 1991) as well as Syria, Egypt and Italy (Balmas, 1994).

Commonly, it is known that several soil- borne fungi are involved in causing crown rot of wheat in Iran. For example, Mansoury (1995) isolated different species of Fusarium, Dreocheclera and Sclerotium from the affected wheat fields in the Fars province. On the other hand, F. culmorum, F. avenaceum and F. acuminatum infections were correlated with yield
losses (Ravanloo and Banihashemi 1999). Other fungi, including Rhizoctonia solani, R. cerealis, F. graminearum and Gaeumanomyces graminis have also been associated with seedling blight and common root rot of wheat in north Iran (Froutan, et al. 1995). Crown rot disease usually causes yield losses under dry conditions in spring when infection of the crown or stem tissue occurs near the soil surface. Initial infections of plants are facilitated by wet conditions but the fungus grows rapidly through the plant tissues when the plants are moisture stress (Wallwork 1996). The present study has been carried out to assess the incidence of crown rot disease of wheat by F. pseudograminearum and the contribution of environmental conditions and susceptible varieties on the increase of its incidence in northwest Iran.

Materials and methods
The study covered wheat fields in the Qazvin, Zanjan, East Azarbyjan and Ardabil provinces from 1999 to 2001. All these areas were visited and plants with symptoms of chlorosis withering, growth reduction, crown necrosis, white heads and blighting of spikelets were collected and transferred to the laboratory of plant pathology in Zanjan University. Six samples infected with crown rot were collected from each province each year and totally 72 complete samples were used to isolate and identify the causal agents. Various fractions of the samples i.e. root crowns, and soils around the roots were cultured in different media. Potato Dextrose Agar (PDA) as common rich medium, Peptone PCNB Agar (PPA) as selective medium and Carnation Leaf Agar (CLA) used as a natural substrate.

Isolated cultures were incubated in a room lighted with near-ultraviolet wave lengths (black light tube, Philips TL 36 w/80 RS F40 BLB) and fluctuating temperatures regime, 25°C during day and 20°C at night under 12 h photoperiod. Sporulation and pigmentation of Fusarium species are favored by this situation (Burgess et al., 1994). Soil dilution technique (Saremi, 1998) was also used to isolate inoculums from soil in the root zone, infected with crown rot disease.

Normally, the causal agent of crown rot disease was cultured on PPA medium for inoculum preparation and then a plug of mycelium was put into chaff-grain medium. This medium was prepared from a mixture of cereal chaff and ground cereal grain (ratio 5:1) used together. The chaff-grain mixture was first soaked in water overnight at 5°C to leach phenolic compounds, then drained before distributing in polyester oven bags. The containers were sealed with a large cotton wool plug and autoclaved for 15 min each on two days. The containers were inoculated with mycelia suspension and incubated under standard conditions, then was air dried after colonization with mycelium, crushed and sieved to the required mesh size for addition to soil for pathogenicity test.

Yield Loss of wheat was assessed at one location of infected fields from each province. Grain yield from 1m² area in each infected field was compared with the plot of same size in a non-infected field. Yield of four wheat fields infected where mostly crown rot disease was assessed in the areas under study. Annual infection rate with percentage of damage for each province was also determined.

Results
Symptom of the disease in wheat
The main symptoms of common root rot and crown rot caused by F. pseudograminearum in the areas studied were yellowing, growth reduction and white head (Fig.1a). The diseased plants were mostly stunted and the symptoms were most striking near or below the surface.
They include brown spots, blotches and rotting on the crown, roots and subcrown internodes (Fig. 1b). The infected plants were stunted, necrotic or rot on rootlets and engulfed in a mass of white fungal growth. Some infected plants showed pink coloration around the crown in studied areas (Fig. 1c). The disease appeared when the fungus was able to build up sufficient inoculum in the soil over two or more years on susceptible varieties.

Isolation of causal agents and other fungi
Generally, 160 fungal isolates were identified during the 3-years study in different wheat fields. Most of the soil-borne fungi that caused common root rot and crown rot were isolated from the fields under studied. Fungi such as Rhizoctonia solani and Drechslera sp., were also found in rare situations. But the major fungal pathogen, isolated with high population, was Fusarium pseudograminearum. Cultures of the fungus formed uniform dense mycelium in PDA medium and microconidia were absence (Fig. 2a). The mycelium was predominantly light yellow, grayish rose at the periphery and white at apex of the colony.

There was significant difference in the frequency of isolations of F. pseudograminearum and other isolated species at either studied site in any year. The incidence of crown rot was more in the relatively drier provinces of Ardabil, East Azarbyjan, Zanjan and Qazvin, which had lower rainfall than other northern provinces, such as, Golestan and Mazenderan. These two humid provinces are close to the Caspian Sea and receive heavy rainfall in the crop season (April and May), conditions suitable for the development of head blight disease. In this situation, Fusarium graminearum (Group 2) was isolated from the head blight affected wheat samples collected only at some locations. This population produced perithecium with ascospores.

Total eight species were isolated from all the regions but F. graminearum G2 was isolated in rare cases in the humid regions only. The infected plants had immature heads and some spikes appeared prematurely bleached. Crown rot caused severe damage in the Ardabil province when dry condition followed infection of plants under wet weather in spring.

Frequency of isolated fungi
The population of F. pseudograminearum was mostly recovered from the sub-crown internodes and roots of the plants suspected to be diseased. Its frequency was 51% among the soil-borne fungi isolated in the areas under study. The frequency of the other fungi was 21% Rhizolania solani, 3% R. cerealis, 7% F. culmorum, 10% Drechsera sp., 3% for Sclerotium rolfsii, and 5% Bipolaris sp.

Rate of yield losses
Crown rot disease caused white heads and resulted in poor seed filling, leading to significant yield losses. Plants of infected fields were compared with noninfected plants in each province. Investigation showed that there were differences in the extent of yield loss in different areas (Tab. 1).

Discussion
The incidence of crown rot and head blight of wheat were significantly affected by environmental factors, mainly rainfall and susceptible varieties. The effect of climate, especially rainfall and temperature, on the abundance of Fusarium species has been reported by various investigators (Burgess et al. 1988; Marasas, et al., 1988; Sangalang et al. 1995). The incidence of Fusarium graminearum (Group 2) in humid areas supported the contention that F. compactum was isolated only from warmer sites and F. sambucium was recovered only from temperate to cold areas (Saremi et al. 1999).
It is important to develop resistant cultivars through breeding effort by applying recurrent selection. It was obvious that using susceptible cultivar such as “Flat” and "Golestan" (local name) resulted in high epidemic of head blight disease in north Iran (Golestan Province). Some cultivars were less infected due to physical barriers. There was difference in the incidence of head blight among cultivars growing in adjacent fields in north Iran (Etebrian and Torabi 1996). Different methods to find sources of resistance to head blight in Iran were reviewed by Allizadeh et al. (2001). Some physiological and morphological characters in wheat were associated with disease resistance. Some varieties with partial resistance showed less reduction in grain yield due to light blight infection. Cultivars also differ in resistance to the crown rot disease, and the more resistant ones should be cultivated over large areas. However the incidence of crown rot disease was mostly correlated with stubble management as the fungus survives in the infected residues (Wearing and Burgess 1977). Burgess et al. (1993) determined the consistency of the effects of stubble management on crown rot disease over longer periods of continuous wheat cultivation and on the increase of disease incidence. The experience showed that stubble retained in some plots enhanced infection of crown rot disease. It was observed that in the Zanjan province the development of crown rot disease was more in poor soils than in soils rich in organic matter content. It has been reported that the soil-borne pathogens are generally less than fungal saprophytes in the soil with high organic matter content (Van Bruggen, 1995). However, crown rot was more on deep heavy clay soils with continuous wheat cultivation. Since crown rot and head blight have become major diseases in the areas of continuous wheat cultivation in Iran, particularly in the northern wheat belt, it is important to control them through management practices and use of tolerant cultivars. Crop rotation, stubble burnt in autumn and careful selection of nitrogen dose can help to minimize the incidence of disease. We have to stop the practice of stubble retention, which leads to significant increase in the incidence of crown rot caused by *F. pseudograminearum*.

**Acknowledgments**

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### Tables

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**Table 1:** Yield losses in wheat in four provinces in northwest Iran caused by *Fusarium pseudograminearum*

### Figure Legends

**Figure 1:** Symptom of crown roon wheat caused by *Fusarium pseudograminearum*, white head (a), crown rot (b) and discoloration of crown (c) on infected plant.

**Figure 2:** Colony morphology (a) and macroconidia (b) of *Fusarium Pseudograminearum* isolated from infected wheat.
Study on Bakanae disease of rice and evaluation of cultivars in Gilan and Zanjan provinces, Iran.

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Abstract
Crown and root rot of rice is one of the important fungal diseases of rice in Gilan and Zanjan provinces, Iran. Plants and soil around the roots of infected rice plants were collected and used to identify the causal agent. Root and crown parts were surfaced sterilized with sodium hypochlorite and then cultured on PDA, PPA and CLA media. Soil samples prepared in water agar were used to isolate the pathogen. The causal agent was identified Fusarium moniliforme as a soil borne fungus. Colonies were initially white but turned violet to grey late. Microconidia were arranged in chain and macroconidia were cylindrical and long with 3-5 septa. The disease was severe in Zanjan province particularly along Ghezel Ozan river where the infection ranged from 70-80%. Root and crown rot was more prevalent in areas where Champa and Gerdeh were being cultivated continuously. Persistent cultivation of rice and seed sowing method intensified disease development and caused significant economic losses. Binam cultivar was the best resistant to the disease in studied areas.

Keywords: Crown, Fusarium moniliforme, Resistance, Rice, Root, Rot

Introduction
Rice (Oryza sativa L.) is the most important agricultural crop of north in Iran. Root rot and crown rot is a significant disease of rice and caused most yield losses in various parts of the country. Generally the increased incidence and economic importance of the disease has been linked to environmental conditions such as humidity and use of susceptible varieties. The disease acquires economic proportions in the northwest of Iran, especially in Gilan and Zanjan provinces. It is also a serious problem in other countries including Japan, Taiwan and Thailand (Nelson et al. 1981). Bakanae disease of rice, also called foot rot in India, occurs widely in Asia and sporadically in other areas of rice production. The most common symptom of disease in some countries may be the elongation of the plant stems. However, the disease may be called “Bakanae“ which is a Japanese word meaning bad seedlings (Sun and Snyder, 1981).

In addition the disease has been reported from the rice tracts of South Asia and European countries (Saremi, 2000). It has been reported that several soil- borne fungi are involved in causing crown rot of rice in Iran. For example, Khosravei (1999) isolated different species of seed borne fungi especially Fusarium species from the affected rice fields in the Mazandran province.

Crown and root rot of rice usually causes yield losses under wet conditions in spring when infection of the crown or stem tissue occurs near the soil surface. The present study has been carried out to assess the occurrence of root and crown rot disease of rice by F. moniliforme and the contribution of factors on the increase of its incidence in northwest Iran.

Materials and methods
Sample collection
The study covered rice fields in the Gilan and provinces from 1999 to 2004. All these areas were visited and plants with symptoms growth reduction, crown necrosis, white heads and
stem elongation were collected and transferred to the laboratory of plant pathology in Zanjan University. Different samples infected with crown rot and root rot were collected from each province each year to isolate and identify the causal agents. Various fractions of the samples i.e. roots, crowns, and soils around the roots were cultured in different media.

**Incubation**

All cultures were incubated in a room lighted with near-ultraviolet wave lengths (black light tube, Philips TL 36 w/80 RS F40 BLB) and fluctuating temperatures regime, 25°C during day and 20°C in night under 12 h photoperiod. Sporulation and pigmentation of *Fusarium* species are favored by this situation (Burgess et al., 1994). Soil dilution technique was also used to isolate inoculums from soil in the root zone suspect to be infected with crown rot disease.

**Media**

Collected samples were cultured in PDA (Potato, Dextrose, and Agar) as common medium, PPA (Peptone, PCNB, and Agar) as selective medium and CLA (Carnation, Leaf, and Agar) as natural medium after surface sterilization with sodium hypochlorite. Cultures were kept in light room with fluctuating temperatures (25°C during day and 20°C at night) and UV lights (12 h light and 12 h dark) to allow the colonies of the pathogen to grow (Burgess et al. 1994). The soil dilution method (Saremi 1998) was used to isolate pathogen inoculum from the soil around the roots of the wheat plant suspected to infected crown rot disease. Pure culture was obtained from each isolate using the single spore culture method and all isolates were identified (Burgess et al.1994; Saremi 1998).

**Soil dilution plate technique**

The technique involved the uniform dispersion of 1 ml of soil suspension of infected field across a selective medium such as PPA. Actually, one gram of infected soil was added to the water agar to produce soil suspension. Propagules in the soil sample suspension germinated within 2-3 days on PPA and produced small colonies by 5-7 days. The suspension was uniformly dispersed over the medium by carefully pipetting 1 ml of soil suspension onto the medium on one edge of the PPA. The plate was then held with a slight slope away from the suspension and gently shaken at right angles to the slope. The suspension slowly spread across the plate with a uniform wetting front.

**Pathogenicity test**

Pathogenicity test was occurred in naturally infected field in Zanjan province. The population density of causal agent was high, with 1575 colony forming propagules unite in one gram soil (CFU g⁻¹ in 0 to 10 cm depth) in some studied areas. By the way, ten varieties of rice including Binam, Kadous, Shafagh, Sahel, Fajr, Khazar, Neda, Nemat, Gerdeh and Champa were cultured in naturally infected soil. Rice cultivars were also cultured in nearly non infected field to obtain the effect of reduction in population density of the fungus to yield loss. The population density of *F. moniliforme* in this field was low with 145 CFU g⁻¹ which nearly healthy crop can be grown. The rate of infection and yield loss production of both fields were generally compared to determine the relatively resistant or sensitivity of varieties in studied area.

**Results**

**Symptom of disease**

Generally various fungal species produced different symptoms on the infected rice plants. However, the main symptoms of common root rot and crown rot in the areas studied were yellowing, stem elongation, pink coloration around the crown, and white head caused by *F. moniliforme* (Fig.1a). The diseased plants were mostly stunted and the symptoms were most
striking near or below the surface. They include brown spots, blotches and rotting on the crown and roots. Some of the infected seeds had not growth and showed rot before emergence in different locations (Fig 1b). This sort of disease was mostly severed in areas that seeds were cultured instead of using seedlings such as growers in Zanjan province. Other soil-borne fungi such as Rhizoctonia solani were also found in rare situations. The fungus leads to formation of white heads with little or no grain. The disease appears when the fungus is able to build up sufficient inoculum in the soil over two or more years on susceptible varieties.

Morphology of causal agent
During the 4-years study in different rice fields various fungal isolates were identified. All the soil-borne fungi that cause common root rot and crown rot were mostly isolated from the studied fields. The incidence of crown rot was more in the relatively humid locations in Zanjan and Gilan provinces. These two humid provinces especially Gilan are close to the Caspian Sea and receive heavy rainfall in the crop season (April and May). The main pathogen was *F. moniliforme* that formed floccose mycelium and became grayish violet or grayish magenta with age (Fig 2a). Microconidia were formed abundantly in chains from monophialide on branched conidiophores or from monophialide formed directly on the hyphae (Fig. 2b).

Nearly resistance varieties
Crown rot disease on rice resulted in poor seed filling, leading to significant yield losses. Plants of infected fields were compared with noninfected plants. Investigation showed that there were differences in the extent of yield loss in different areas (Fig 3). Inoculum density of *Fusarium moniliforme* could affect the incidence of disease. High population density of the pathogen has been associated severity of crown rot and yield production of rice in studied area. “Binam” had the most yield production and less infected disease and “Champa” showed low yield production in the field (Fig 3). However, the result showed there was an obvious difference between the infection rate and yield loss of varieties in studied area. Binam was the main resistant race in the field, however Kadous and Shafagh also showed better resistance than Nemat, Neda and Khazar races. Other varieties such as Fajr, Sahel and Shafagh were relatively resistant to the disease, any how they showed reduced crop production.

Rate of yield losses
Root rot and crown rot disease caused white heads and resulted in poor seed filling, leading to significant yield losses. Plants of infected fields were compared with noninfected plants in each rice cultivar. Investigation showed that there were differences in the extent of yield loss in different cultivars. Binam had lower infection (%7) but Champa (local name) showed the highest (%38) infection in natural infected soil (Tab. 1).

Discussion
Commonly, the incidence of crown rot and root rot of rice were significantly affected by environmental factors, mainly rainfall and susceptible varieties. The effect of climate, especially rainfall and temperature, on the abundance of *Fusarium* species has been reported by various investigators (Sangalang et al. 1995; Burgess et al. 1996; Marasas et al., 1988). The incidence of *Fusarium moniliforme* in humid areas supported the contention that *F. compactum* was isolated only from warmer sites and *F. sambucium* was recovered only from temperate to cold areas (Saremi et al. 1999).
It is important to develop resistant cultivars through breeding effort by applying recurrent selection. It was obvious that using susceptible cultivar such as “Gerdeh” and "Champa" (local name) resulted in high epidemic crown rot disease in Zanjan province.

Some physiological and morphological characters in rice may also associate with disease resistance. Study also showed that some varieties with partial resistance had less reduction in yield due to light infection. For example, Binam, Kadous, Shafagh and Sahel varieties were more resistant than other varieties such as Namat, Neda and Khazar. Namely, more resistant ones should be cultivated over large areas to increase high production.

There was an investigation by Padasht et al. (1996) to find suitable fungicide for controlling crown rot disease on rice in north Iran. Of course chemical control may have some negative effect on other soil microorganisms or produce resistance of the fungal pathogen. However, it has been reported that Initial greenhouse studies showed the treatment of infested seed with the fungicide Maxim resulted in a significant reduction of diseased plants in some places (Nyvall, 1999). Further study, especially field tests, are needed to determine the effectiveness of fungicide seed treatments under field conditions, possible phytotoxicity resulting in inhibition of root elongation, and potential impact on stand establishment.

Since the disease was described as widespread, occurring in various counties and also spreading to worldwide locations. Scientists took a deeper look into the life cycle of fungus caused crown rot disease on rice in different countries to find the best way to control the pathogen.

However the incidence of crown rot disease on grains caused by Fusarium species was mostly correlated with stubble management as the fungus survives in the infected residues (Wearing and Burgess 1977). Burgess et al. (1993) determined the consistency of the effects of stubble management on crown rot disease over longer periods of continuous crop cultivation and on the increase of disease incidence. The seedlings of rice may become infected from the pathogen Fusarium moniliforme in soil or residue but all available information indicates the disease is primarily seed born. The pathogen is abundant in residue of harvested plants, providing the main source for infestation of seed. Studies have shown that the pathogen is primarily a surface contaminant of seed.

It was observed that in the Zanjan province the development of crown rot disease was more in poor soils than in soils rich in organic matter content. It has been generally reported that the soil-borne pathogens are less than fungal saprophytes in the soil with high organic matter content (Van Bruggen, 1995). However, crown rot was more on deep heavy clay soils with continuous rice cultivation.

Since crown rot and root rot of rice have become major diseases in the areas of continuous rice cultivation in Iran, particularly in the northern rice belt, it is important to control them through management practices and use of tolerant cultivars. Crop rotation, stubble burnt in autumn and careful selection of nearly resistant varieties can be helpful to minimize the incidence of disease. We have to stop the using continuously one variety such as “Gerdeh” or “Champa” which leads to significant increase in the incidence of crown rot caused by F. moniliforme in Zanjan province.

Acknowledgments
The study was supported by the Iranian Center of National Projects under the project No 21205 "Crown rot and root rot diseases of rice and its ecological control in northwest of Iran" which gratefully acknowledged.

References:
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Table 1: Yield losses in four cultivars of rice crop in Zanjan province, Iran caused by *Fusarium moniliforme* in natural infected soil.

Figure Legends

Figure 1: Elongation and white head (a) and seed rot of rice (b) caused by *Fusarium moniliforme* (b) in Zanjan province.

Figure 2: Colony morphology on PDA medium (a) and long chain of Microconidia (b) of *Fusarium moniliforme* the casual agent of rice rot.
Figure 3: Cultivation of different rice varieties in infected soil (a) and white head disease due to infection by *F. moniliforme*.
Implementation of an indirect approach to computing (co)variance of Mendelian sampling for a marked quantitative trait locus

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Abstract
Several researchers have discussed computing techniques for the inverse of the gametic relationship matrix (\(G^{-1}\)), which is required for QTL analyses using the mixed model methodology. In order to obtain \(G^{-1}\), a block diagonal matrix (\(D\)) that is proportional to the conditional covariance matrix of Mendelian sampling is needed. In this study, implementation of an indirect method is described for obtaining \(D\) matrix. In the current approach, elements of \(D\) matrix for animals having the same sire and the same pseudo-generation number are computed in one back and forth exploration of the pedigree. The performance of the current approach was compared with that of adaptation of the tabular method, or a direct approach, using two simulated populations (200 or 400 base sires) with 40 discrete generations. A situation where one marker and one QTL are located 5 cM far apart was considered. Marker genotypes were assumed to be known. When 40 years were evaluated, computation time of the current indirect approach was 79 and 60\% relative to the direct approach for the populations with 200 and 400 base sires, respectively. In this case, the memory requirements for the indirect and the direct approaches were 13.5 and 436.6 Mb for the population with 200 base sires and 13.5 and 862.0 Mb for the population with 400 base sires, respectively. It is shown that for computation of \(D\) matrix, the indirect approach could not only be faster, but also require significantly less memory, than the direct approach.

Key Words: gametic relationship matrix, indirect method, marked QTL, Mendelian sampling, tabular method

Introduction
The inverse of the gametic relationship matrix (\(G^{-1}\)) is required for QTL analyses using the mixed model methodology. For a marked QTL, several researchers have discussed computing techniques to obtain \(G^{-1}\) directly without forming \(G\) itself (e.g., Fernando and Grossman, 1989; van Arendonk et al., 1994; Wang et al., 1995; Abdel-Azim and Freeman, 2001). The \(G^{-1}\) could be constructed directly by using the partitioned matrix theory (van Arendonk et al., 1994) or making use of the decomposition of \(G\) similar to that of numerator relationship matrix described by Henderson (1976). In both the approaches, a block diagonal matrix (\(D\)) that is proportional to the conditional covariance matrix of Mendelian sampling is required. Abdel-Azim and Freeman (2001) extended an adaptation of the tabular method by Tier (1990) to obtain conditional probabilities of identity-by-descent (IBD) or conditional relationships within animals and between their parents and then \(D\) matrix for a marked QTL. With the algorithm of Tier (1990), or a direct method, a small subset of \(G\) is required to compute its diagonal. This algorithm is quick, because it traces the pedigree only twice, but needs relatively large memory to store the subset of \(G\). Recently, Colleau (2002) presented an interesting indirect method for the extensive computation of the relationships, assuming known inbreeding coefficients, and also described an implementation of the indirect approach to obtaining inbreeding coefficients.
In this study, we implemented Colleau’s (2002) indirect method to compute conditional relationships and inbreeding coefficients for a marked QTL which are required to calculate the conditional variances and covariances of Mendelian sampling. The computation time and the memory requirement of the indirect approach were compared with those of a direct approach using the tabular method.

**Materials and Methods**

The conditional covariance matrix of Mendelian sampling for animal \( i \) is:

\[
\text{Var}(\mathbf{M}_i | \mathcal{M}) = \mathbf{D}_i \sigma_i^2,
\]

where \( \mathbf{M}_i \) is the \( 2 \times 2 \) Mendelian sampling-matrix, \( \mathcal{M} \) stands for marker genotypes, \( \sigma_i^2 \) is the additive genetic variance for the marked QTL, and as shown by Wang et al. (1995), \( \mathbf{D}_i \) can be calculated as:

\[
\mathbf{D}_i = \mathbf{G}_{ii} - \begin{bmatrix} \mathbf{B}_i^s & \mathbf{B}_i^d \end{bmatrix} \begin{bmatrix} \mathbf{G}_{s,i} & \mathbf{G}_{s,d} \\ \mathbf{G}'_{s,i} & \mathbf{G}'_{s,d} \end{bmatrix} \begin{bmatrix} \mathbf{B}'_i^s \\ \mathbf{B}'_i^d \end{bmatrix},
\]

where \( \mathbf{G}_{ii}, \mathbf{G}_{s,a_i}, \mathbf{G}_{d,d_i}, \) and \( \mathbf{G}_{s,d_i} \) are \( 2 \times 2 \) matrices containing the conditional relationships, and \( \mathbf{B}_i^s \) and \( \mathbf{B}_i^d \) are \( 2 \times 2 \) matrices containing the conditional probabilities that QTL alleles in animal \( i \) descended from its sire or its dam (PDQ), respectively. Wang et al. (1995) described the details for the calculation of PDQs for a QTL linked to a marker. The \( \mathbf{G}_{ii} \) could be easily derived from \( \mathbf{G}_{s,d_i} \), therefore the main task would be to compute \( \mathbf{G}_{s,d_i} \).

The indirect algorithm of Colleau (2002) can be applied to compute \( \mathbf{G}_{s,d_i} \). Colleau (2002) explained a method to multiply the numerator relationship matrix corresponding to planned matings by any vector. He showed that the relationships between a sire and his mates could be obtained simultaneously, if their progeny have the same pseudo-generation number. Here we use his method at gametic level in order to compute \( \mathbf{G}_{s,d_i} \), as follows:

\[
\mathbf{G}^* \mathbf{X} = \mathbf{Y},
\]

where \( \mathbf{G}^* \) is the gametic relationship matrix involving the sire of interest, his mates and their ancestors, \( \mathbf{X} \) is a matrix containing \( 2 \times 2 \) identity matrix at the position of the sire and \( 2 \times 2 \) null matrices for others, and \( \mathbf{Y} \) is a matrix containing the IBDs between the sire and other animals considered. After the decomposition of \( \mathbf{G}^* \), we have:

\[
(\mathbf{T}^{-1})' \mathbf{D}'^{-1} \mathbf{T}'^{-1} \mathbf{Y} = \mathbf{X},
\]

where \( \mathbf{T}^{-1} \) is a lower triangular matrix whose elements are zeros except for 1 on the diagonal and \( -\mathbf{B}_i^s \) and \( -\mathbf{B}_i^d \) for elements in which sires and dams and their progeny are linked, respectively, and \( \mathbf{D}'^{-1} \) is a block diagonal matrix whose diagonal matrices are \( \sigma_i^2 / \text{Var}(\mathbf{M}_i | \mathcal{M}) \) for the sire of interest, his mates and their ancestors. Animals were assumed to be sequentially numbered from 1 to \( n \), so that parents precede their progeny, and \( \mathbf{D}' \) matrix was supposed to be known.

The above system can be solved for \( \mathbf{Y} \) by the following steps using the special characteristics of \( \mathbf{T}^{-1} \) matrix (Colleau, 2002):

1) Solve \( (\mathbf{T}^{-1})' \mathbf{Z} = \mathbf{X} \) for \( \mathbf{Z} \) by tracing forth the pedigree, and
2) Solve \( \mathbf{T}^{-1} \mathbf{Y} = \mathbf{D}' \mathbf{Z} \) for \( \mathbf{Y} \) by tracing back the pedigree,
where $Z = D^{-1}T^{-1}Y$. With this indirect algorithm, the conditional relationships between a sire and his mates that have progeny with the same pseudo-generation number can be computed in one back and forth exploration of the pedigree simultaneously. Finally, the $2 \times 2$ $D$ matrices of progeny can be obtained from these conditional relationships and PDQs.

In order to compare the performance of the current indirect approach with that of the direct approach based on Tier’s (1990) method, two populations with 40 discrete generations were simulated. A situation where one marker and one QTL are located 5 cM far apart was considered. Marker genotypes were assumed to be known for all animals. The 25 percent of genetic variation was represented by the QTL. Base population consisted of either 200 or 400 sires, and 1000 dams. Sires and dams were mated at random, each dam produced 4 progeny, and selection was based on phenotype. The two approaches were programmed using C++ and run on a PC (Pentium 4; 3.2 GHz processor) under Windows 2000.

**Results**

Computer requirements for the two approaches are presented in Table 1. In the simulated populations, the indirect approach was obviously faster than the direct approach. Computation time for the indirect approach, relative to the direct approach, ranged from 29.9 to 78.8%. Both the direct and indirect algorithms became slow, when the numbers of sires and generations increased. Memory requirements for the indirect algorithm were found to be within the range from 1.6 to 6.5% compared to the direct algorithm. Hence, the required memory with the indirect algorithm was drastically low relative to the direct algorithm in all situations. For a given number of generations, the number of required elements of $G$ matrix (for the direct algorithm) was always larger in the population with 400 base sires than in the population with 200 base sires.

**Discussion**

An implementation of an indirect method to computing the conditional (co)variance of Mendelian sampling for a QTL linked to a marker has been presented. The efficiency of the current indirect approach depends remarkably on the number of sires, since progeny of each sire are evaluated at a time. In the situation of overlapping generations, however, all progeny of a sire might not be evaluated at a time, as they could have different pseudo-generation numbers. We are now working to solve this problem. In practice, the number of sires is usually less than that of dams, but dams and sires can be exchanged, if the number of dams is less. The computation time and the memory requirement of the direct approach employing Tier’s (1990) method depend on the number of required elements of $G$ matrix. In the case of many generations and small family size, the subset of $G$ matrix which is necessary to compute its diagonal is likely to be large. It is shown that the indirect approach could not only be faster, but also require significantly less memory, than the direct approach. Using PDQs of animals and calculated $D$ matrix, $G^{-1}$ can be constructed easily by using the partitioned matrix theory or making use of the decomposition of $G$. The extension of the current indirect approach as well as the direct one to situations where one QTL linked to more than one marker would be straightforward, assuming known PDQs for animals.

**References**


**Tables Legends**

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Preparation of Salmonella typhimurium killed vaccine by Gamma irradiation

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Abstract

After culture and multiplication of Salmonella typhimurium (1735) at 37°C, the characteristics of the bacteria considered and the growth curve was drawn. The best time for going out the bacteria from 37°C was 8 hours, after incubation. Because the bacteria has been in the logarithmic phase yet. The first stage the purified bacteria in PBS were irradiated by gamma ray from 60Co Source in different doses (2, 2.5, 3, 3.5, 4 and 5kGy). The optimum dose of gamma ray for inactivation of S. typhimurium (1735) was obtained 3.5kGy.

The three stocks of Salmonella typhimurium radio-vaccines in different doses (3.5, 4 and 4.5kGy) and one stock of the killed vaccine by formaline were prepared, and the sterility tests were done for all of them.

In the second stage the LD50 test was done for the salmonella serotype and the radiovaccines were inoculated to suitable laboratory animals. Finally all vaccinated animal received challenge injection of virulent bacteria. In the third stage we tested some possible harmful effects of vaccine on inoculated animal pathologically. The results of safety test revealed that there is no disadvantage in application of radio vaccine.

Key Words: Radiovaccine, Salmonella, Typhimurium, Irradiation

Introduction

Salmonella is the agent of salmonellosis disease. Salmonellosis is an enzootic disease that causes septicemia abortion, arthritis and intestine inflammation.

There are two types of Salmonellonella vaccines: 1) attenuated vaccine, 2) killed vaccine. The preparation of killed vaccine by exposing suspensions of microorganisms to ionizing radiations has been reported (Gordon et al. 1964, Jordan et al. 1956).

Irradiation has been found to be useful in preparing of vaccine against Salmonella (Previte, 1968).

There are three stages during of vaccine production processing: 1) sterility test, 2) potency test, 3) safety test.

The conventional methods used for the preparation of typhoid vaccine involve inactivation of bacteria by acetone, heat-phenol or formalin (Felix 1952). However, the endotoxin of Salmonella is not detoxified by these treatments, thereby inducing side-reactions. Nerkar and et al reported (1977) that irradiation not only killed the bacteria (S. typhimurium) but also resulted in significant reduction in the toxicity of endotoxin.
Death of microorganisms from exposure of population to ionizing radiation is Logarithmic in nature that makes Dose/ survival curves are straightlines (Aram et al.1978).

**Material and Methods**

Salmonella typhimurium (1735) was obtained from microbiological department of vaccine and serum Institute of Razi,IRAN, as lyophilized bacteria. It was grown for 18 hours in Tryptic Soy Broth (TSB) at 37°C. Then it was grown on Maccacy Agar and Nutrient Agar media and gram staining slides were prepared. The bacteria were inoculated on 300 ml of TSB as the main medium. The growth curve of S.typhimurium (1735) was drawn by measuring OD(Optical Density) at 600nm Length wave by Spectrometric method , and the best time to go out the bacteria from 37°C was found , when the bacteria was in logarithmic phase.

At first the bacteria suspension in PBS (pH=7/2) equal of McFarland Standard No: 1(3×10⁸ particles/ml) was prepared in four containers (each one was 50ml). Then the bacteria suspensions were irradiated at 2,3,4 and 5KGY by Gamma cell (Model Issledovapel, PX-30) with dose rate :0,932 Gy/Sec and activity: 6180 Ci (60- Co Source). Then the irradiated bacteria suspensions were grown on TSB , Tyoglocolate(TYC), Maccancy , Nutrient agar and Triple Sugar Iron agar.

Again the bacteria suspensions ( 2,1×10⁹ particls/ ml ) were prepared and divided into four containers (each one 50ml). Three of them were irradiated at 3.5 ,4.5 , and 5.5 kGy and the fourth suspension was treated by formalin ( 4/1000 ) in 37°C at 24 hours.

Then the sterile Al (OH) 3 as an adjuvant was added to the suspensions (% 10). All of the suspensions were grown on TSB and Tyo to ensure the sterility of vaccine. TheLD50 of the bacteria was determined before irradiation that it was 35 bacteria/ml. Agglutination test was done between Salmonella Ag and Serum of the mice and the stool of these animals were cultured by selenit-F-Broth, Maccancy Agar, TSI and Urea Agar. We had 5 groups of mice, 4 vaccine groups and one control group, each group containd 14 mice. One ml of each vaccine was injected to each mouse interaperitaneally.Also we had another 5 groups of mice and injected vaccines subcutancously. After 28 days, the vaccines were injected again and after 14 days the second injection were done. The challenge of vaccine groups with pathogenic type of Salmonella typhimurium 280 bacteria/ml was done. Then the LD50 test of challenged groups was done by 35, 120, 230 bacteria/ml concentration. Finally the killed mice after challenge were anatomized and their Liver, Spleen, Intestine and Kidney were studied pathologically.

Safety test was done by 5 groups of rabbits which haven’t exposed by salmonella yet, each group contains 4 rabbits. At first the agglutination test was done between salmonella Ag and the rabbit serum. The vaccines were injected subcutaneously, 2 weeks later the second injection was done, after one week was blooded from the rabbit’s heart. The rabbit serum separated and diluted 1/20, 1/40, 1/80 and 1/160 for doing agglutination test.

**Results**

According to the growth curve of Salmonella typhimurium, the best time for irradiation was 8 hours after inoculation of bacteria at37°C, because the bacteria were in the Logarithmic phase(Figure 1).
About the mice groups which were injected interaperitonally, there were 8 killed mice in the control group; however there was not any killed animal in the vaccine groups after challenge.
Also about the groups who were injected subcutaneously, there were 12 killed mice in the control group and 2 killed mice in irradiated vaccine groups and 2 killed mice in formalin vaccine group (Table 1).
Results of anatomizing the killed animals indicated that necrosis of Liver and liverish, Splints were the main reasons for death of mice.
The results of agglutination tests for rabbit serum after safety test are in( table 2). According to statistic analysis, the vaccines and control groups were different from each others, these different due to type of vaccine.

Discussion
Nerkar DP, et al (1977) in Biochemistry and food technology Division, Bhabha Atomic Research center India reported Gamma-irradiation of S. typhymurium cells up to a dose of 500 Krad significantly reduces their toxicity. However, the antigenecity of these cells is not altered, which suggests that these cells could be used as a vaccine. The protection offered by the irradiated cells is comparable to that of formalin-treated cells.
Also in this research both of the irradiated and formalined vaccines are safety. Also after agglutination test the irradiated vaccines by 3.5, 4.5 and 5.5 kGy were positive till 1/80 and 1/20 dilutions, respectively. The formalined vaccine was negative in agglutination test.
According to the table 1, the best immunization was made by irradiated vaccine, 3.5Kgy and other irradiated vaccines (4.5 and 5.5Kgy) did not make more immunization.
Also the Formalin vaccine was weaker than irradiated vaccines. Therefore the Optimum dose of gamma ray for inactivation of S.typhimurium was obtained 3.5Kgy.

References
Previte JJ (1968) J. Bact., 95,2165
Table 1: challenge with 1ml pure suspension dilution 1500CUF

<table>
<thead>
<tr>
<th>groups</th>
<th>first group radio-vaccine 3.5KGy</th>
<th>second group radio-vaccine 4.5KGy</th>
<th>third group radio-vaccine 5.5KGy</th>
<th>forth group vaccine with formaline</th>
<th>control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>mice</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>killed</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
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</table>

Table 2: The results of agglutination testes for rabbit serum after safety test

<table>
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<tr>
<th>groups</th>
<th>serum dilution</th>
<th>1/20</th>
<th>1/80</th>
<th>1/160</th>
</tr>
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<td>first group radio-vaccine 3.5 KGy</td>
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<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>second group radio-vaccine 4.5 KGy</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>third group radio-vaccine 5.5 KGy</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>fourth group with formaline vaccine</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<tr>
<td>control group</td>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>
Figure 1: Logarithmic growth curve of Salmonella typhimurium

Time (hour)
Absorption dose (600nm) the time after inoculation of bacteria
Present Status of the Powdery Mildew Resistance Gene Er in Pea (Pisum sativum)

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Abstract
Although powdery mildew resistance, identified as a Mendelian trait in 1925, was reported to be under control of a single gene as long back as in 1948 by Harland, uncertainty still prevails as reports of digenic control have been appearing at regular intervals. Our studies over more than two decades involving 151 crosses and segregation analyzed in 41,424 F2 plants recorded only monogenic control of the trait. Molecular mapping with RAPD and SCAR markers also confirmed only one gene, Er, on the 6th chromosome of pea genome. Monogenic control of powdery mildew resistance has also been discovered in other related taxa, such as, P. sativum ssp. arvense and P. fulvum. The second putative gene has not been mapped and its existence can be doubted looking at the contradictory nature of the studies proposing two-gene control of powdery mildew resistance in pea. Close monitoring of the etiology of the disease under different conditions, including hot spots, leads to the conclusion that the two-gene hypothesis is a result of improper identification of disease symptoms and, consequently, erroneous classification of plants in a segregating population.

Introduction
Powdery mildew is a disease of great economic importance for the pea crop all over the world. Heavy infections of Erysiphe pisi cause devastating damage to the crop and reduce grain yield by at least one-third (Sharma, 1995). Although the disease can be controlled very effectively through fungicidal treatment, genetic resistance is a far more safe, reliable, effective and cost-free alternative to ensure a healthy crop. Hammurlund (1925) first described genotypes resistant to powdery mildew from his pea collections. However, the mode of inheritance of powdery mildew resistance (PMR) as a monogenic recessive trait was reported by Harland (1948). Heringa et al. (1969), on the other hand, somewhat vaguely concluded that the trait is under the control of two recessive genes. Reports about multigenic nature of PMR have been appearing till recently (Sokhi et al., 1979; Kumar and Singh, 1981; Singh et al., 1983; Tiwari et al., 1997) even though monogenic inheritance of powdery mildew resistance (PMR) was confirmed earlier (Singh, 1984). These reports were either not convincing or self-contradictory. An overwhelming volume of experimental evidence suggested only monogenic control of PMR. Our own experience of pea research and breeding never gave any indication of more than one gene being involved in PMR. The data generated from a series of Ph.D. programmes (Gupta, 1987; Sarala, 1993; Rakshit, 1997; Kala, 1998; Janila, 1999; Janila et al., 2001; Srivastav, 2004 [Ph.D. thesis under preparation]) and other studies (Sharma, 1995, 2003a, b; Sharma and Yadav, 2003; Sharma, unpublished) provides convincing evidence in favour of PMR being controlled by a single recessive gene. Analysis over the years has also made it possible to understand the nature of fungal growth on the powdery mildew resistant (PMR) and
susceptible (PMS) plants under various environments and also analyze the possible reasons for discrepancies and contradictory reporting on this subject.

**Material and Method**
The parental lines involved in crosses were either established PMR or PMS germplasm accessions and cultivars or wild relatives of the cultivated peas. The crosses were made and the F2 populations were analyzed for PMS–PMR segregation at the Delhi centre of the Indian Agricultural Research Institute and the Summer Wheat Nursery in the Lahaul Valley in high Himalayas which is a hot spot for powdery mildew development. It has been a general observation that even the PMR plants allow a certain degree of fungal growth on their foliage (i.e. leaves and stipules) under certain conditions. However, the infection does not extend to the stem, peduncles and pods even if the leaves and stipules are heavily covered with mycelium (see also Haq et al., 2000). Also, the fungal infection on the PMR plants does not cause tissue mortality, leading to browning of leaf surface. Mechanical wiping of the fungus from the leaves of PMR genotypes exposes healthy tissue. These two criteria allow a perfect distinction between plants that are genetically susceptible or resistant. With this in view, the Lahaul station is also used for confirmation of resistance.

**Results and Discussion**
Conventionally, intensity of disease is recorded on a scale. This approach frequently ends up with quantification of disease incidence and may lead to variable segregation ratios, and even continuous variation. In one of the studies under review (Gupta, 1987), the disease intensity (in fact, fungal growth) was scored on a 5-point scale and the segregants with grade 1 on the scale were taken to be resistant, and those falling under grades 2–5 were treated as susceptible to various degree. The data were analyzed for monogenic, two types of digenic, and also for trigenic segregation (Table 1).

It can be seen that recording disease severity on a quantitative scale does not provide a segregation ratio that would fit into any of the expected patterns. This is because based on the sole criterion of infection on the plants, without taking into consideration the nature of fungal growth and tissue response, they will always be placed in one or the other class of susceptibility. Thus, quantification of infection could lead to variable conclusions about monogenic, multigenic and even quantitative nature of PMR. Since disease scoring on a scale was found to be unsuitable for genetic analysis, in all subsequent studies individual plants in a segregating population were identified as genetically resistant or susceptible on the basis of fungal growth on the stem and pods. The segregation analysis of 151 crosses is presented in Table 2.

Table 1. Segregation for powdery mildew resistance on 1–5 point scale. Grades of disease severity: 1—resistant; 2–5—susceptible with different degree of disease intensity.
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The 41,424 F₂ plants analyzed segregated into 31,222 PMS and 10,202 PMR which fits well the expected 3 R : 1 S ratio ($\chi^2 = 3.05, P = 0.07$). It is noteworthy that the number of PMS plants was slightly more than expected. The possibility cannot be ruled out that a part of this deviation resulted due to counting of a few PMR segregants as PMS based on the appearance of fungus on their leaves and stipules. Careful screening, however, gives a perfect 3 S : 1 R segregation in the F₂ and progeny segregation of 1 S : 2 Segr. : 1 R in the F₃ generation (Sakr and Muehlbauer, 1996). In spite of these minor deviations, the monogenic inheritance of PMR is nevertheless confirmed beyond doubt.

Table 2. Mendelian segregation for powdery mildew resistance in the F₂ populations of different studies

<table>
<thead>
<tr>
<th>Author</th>
<th>No. of crosses</th>
<th>No. of F₂ plants</th>
<th>F₂ segregation</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S     R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M.D. Gupta</td>
<td>78</td>
<td>9173</td>
<td>6911 2262</td>
<td>0.57</td>
<td>0.45</td>
</tr>
<tr>
<td>K Sarala</td>
<td>35</td>
<td>5650</td>
<td>4296 1354</td>
<td>3.23</td>
<td>0.07</td>
</tr>
<tr>
<td>S. Rakshit (F₂ progenies)</td>
<td>2</td>
<td>142</td>
<td>106   36</td>
<td>0.67</td>
<td>0.41</td>
</tr>
<tr>
<td>Rakshit (F₃ progenies)</td>
<td>2</td>
<td>1742</td>
<td>1312 430</td>
<td>0.09</td>
<td>0.76</td>
</tr>
<tr>
<td>P. Janila</td>
<td>10</td>
<td>3135</td>
<td>2356 779</td>
<td>0.04</td>
<td>0.90</td>
</tr>
<tr>
<td>Y.K. Kala</td>
<td>43</td>
<td>19906</td>
<td>14984 4922</td>
<td>0.80</td>
<td>0.38</td>
</tr>
<tr>
<td>B. Sharma (unpublished)</td>
<td>21</td>
<td>1676</td>
<td>1257 419</td>
<td>0.38</td>
<td>0.54</td>
</tr>
<tr>
<td>Total</td>
<td>151</td>
<td>41424</td>
<td>31222 10202</td>
<td>3.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Test-crosses were also carried to confirm the monogenic nature of this trait in which the heterozygous F₁ plants were crossed with their respective recessive parents in 11 crosses (Table 3). The back-crossed populations in all the crosses segregated into 1 R : 1 S ratio. Heterogeneity between the crosses was nonsignificant ($\chi^2 = 4.32$ at 10 d.f.) and the results of all the crosses can be pooled to improve confidence of analysis. The segregation of F₂ populations comprising 391 plants into 201 PMS and 190 PMR was in good agreement of the expected monogenic ratio ($\chi^2 = 0.31, P = 0.41$). The parent genotypes used in these crosses were not related in their pedigree and possibly derived their gene for resistance from different sources.
Table 3. Segregation for powdery mildew resistance in test-crosses [R×S)×R]

<table>
<thead>
<tr>
<th>Test-cross</th>
<th>No. of plants</th>
<th>Ratio 1 : 1</th>
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</thead>
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<tr>
<td></td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>(DMR 11 × Pusa 10) × DMR 11</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>(DMR 11 × Type 163) × DMR 11</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>(Mexique 4×Pusa 10)×Mexique 4</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>(Mexique 4×Type 163)×Mexique 4</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>(Mexique 4×KPSD 1)×Mexique 4</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>(Mexique 4×PG 3) × Mexique 4</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>(Tara × Pusa 10) × Tara</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>(Tara × Type 163) × Tara</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>(HUP 5 × Type 163) × HUP 5</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>(S 143 × Type 163) × S 13</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>(HFP 4 × Type 163) × HFP 4</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Pooled over 11 test-crosses</td>
<td>201</td>
<td>190</td>
</tr>
<tr>
<td>Heterogeneity (\chi^2) (4 d. f.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The variety Mexique 4 used in four test-crosses deserves special mention. This strain from Mexico was used by Heringa et al. (1969) to reach the conclusion that PMR in pea is caused by two recessive genes. In the present study, the crosses made with Mexique 4 did not show any indication of a two-gene carrier. All the crosses involving Mexique 4 as a parent yielded monogenic segregation in the F2 as well as BC populations. This unequivocally proves that Mexique 4 carries a single recessive gene for resistance. Similar results were obtained from test-crosses by Singh (1984) in his Ph.D. programme. Six PMR strains (P 65788, P 386, P 388, T 10, P 185 and Rachna) were crossed with five PMS varieties (T 163, BR 12, L 116, Rau 21 and Sel 23). The back-cross populations of 30 crosses segregated into 818 PMS and 786 PMR, which was in good agreement with the 1S : 1R ratio \((\chi^2 = 0.32, P = 0.7)\).

Complementation tests were also conducted to ascertain whether different donors of PMR carried a common or different genes for resistance. As can be seen from Table 4, the PMR parents involved in the crosses had different origin or were received from different sources, and therefore could not have shared a common gene in a great majority of cases. The results of complementation analysis show that the resistant parents belong to the same complementation group hence carry a common gene. Not a single case of complementation (F1 susceptible) was recorded. Failure of complementation further supports the hypothesis of PMR being a monogenic trait in pea.
Table 4. Complementation test for powdery mildew resistance in pea

<table>
<thead>
<tr>
<th>Cross</th>
<th>F$_1$ plants</th>
<th>F$_2$ plants</th>
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<tr>
<td></td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Male</td>
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</tr>
<tr>
<td>DMR 11</td>
<td>51</td>
<td>1</td>
</tr>
<tr>
<td>Mexique 4</td>
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<td>1</td>
</tr>
<tr>
<td>S 143</td>
<td>38</td>
<td>0</td>
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<td>Tara</td>
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<td>DMR 11</td>
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<td>HFP 4</td>
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<td>HUP 5</td>
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<td>DMR 11</td>
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</tr>
<tr>
<td>Rachna</td>
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<td>Pant P 5</td>
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<tr>
<td>Tara</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Tara</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>P 1422</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>HFP 4</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>HFP 4</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>HUP 5</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>SKL 43</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

— indicates F$_2$ not screened.

The isolated cases of individual F$_1$ and F$_2$ plants recorded as susceptible are possibly a result of outcrossing or erroneous classification due to the presence of fungus on the
foliage of PMR plants under severe infection and favourable conditions for fungal growth. This contention is supported by the fact that PMS plants were recorded in the F2 progenies of 11 noncomplementing crosses and they do not fit into any ratio of genetic segregation. It may also be added that early maturing genotypes catch the infection early in the season and are more heavily coated with fungal mycelium at any point of time. In a cross simultaneously segregating for PMR and maturity duration one is faced with a complex situation which increases the chances of misclassification. This could be a reason for wrong classification of plants in segregating populations under variable conditions, which may lead to F2 ratios other than monogenic. Likewise, Singh (1984) also intermated the six PMR strains mentioned above in diallel fashion to investigate complementation for the PMR trait. The 15 crosses in the study always produced PMR offsprings in the F1 (771 R : 0 S) and F2 (2333 R : 0 S) progenies. Not a single susceptible plant was noticed. Faced with these difficulties, Marx (1971, 1986) mapped the Er gene on chromosome 3 but also found mapping of this gene to be “elusive”. Sakr and Muehlbauer (1996) did not find linkage between Er and 19 morphological and enzyme markers of chromosomes 1, 3, 4, 5 and 7. They, however, reported linkage of Er with Sldh gene on chromosome 7, which has never been confirmed. Our studies have mapped the Er gene on chromosome 6 (Sarala, 1993; Kala, 1998, Janila, 1999; Janila and Sharma, 2003).

The Er gene has also been tagged on chromosome 6 using molecular markers in isolation (Timmerman et al., 1994; Tiwari, 1998) as well as in conjunction with several known morphological markers of this chromosome like Na, Wlo, Arg, Fl, Pl and thiB (Rakshit, 1997; Janila, 1999; Janila and Sharma, 2004). The existence of the second PMR gene (Er2) has never been demonstrated directly from an independent study. Conclusions about its existence are based on indirect indications from segregation ratios, which apparently were obtained due to erroneous classification of segregants in the F2 generation. Tiwari et al. (1997) concluded both monogenic and digenic segregations in the same cross under different conditions of screening.

Our recent studies (Sharma, 2003b; Sharma and Yadav, 2003) have revealed that monogenic control of PMR also operates in other related taxa under the genus Pisum, e.g. P. sativum ssp. arvense and P. fulvum. These observations allow us to extend the hypothesis of monogenic nature of powdery mildew resistance to the entire genus Pisum.

References


Three-gene control of cotyledon colour in lentil (*Lens culinaris*)

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Abstract

Two genes for cotyledon colour in lentil were reported earlier which cause bright yellow, brownish yellow (called brown) coloration of cotyledons in dominant condition. They were assigned gene symbols *Y* and *B*, respectively. The orange (red) cotyledons are produced in dominant homozygous condition of both genes (*YYBB*) and they remain green in the double recessive homozygotes (*yybb*). A third gene blocks the synthesis of both pigments in recessive state and results in deeper green cotyledons hence called “dark green” with gene symbol *Dg*. This gene has recessive epistatic effect on *Y* and *B* simultaneously. It was hypothesized that that *Dg* acts at a stage earlier than *Y* and *B* in the metabolic pathway of pigment synthesis. Segregation ratios under trigenic interactions with recessive epistasis of *Dg* over *Y* and *B* were predicted earlier, which has been confirmed experimentally.

Key Words: Lentil, *Lens culinaris*, cotyledon colour, trigenic control

Introduction

For a long time, the orange (popularly called red) colour of lentil cotyledons was recognized as a monogenic trait (Tschermak-Seysenag, 1928; Wilson et al., 1970; Singh, 1978; Slinkard, 1978; Sinha et al., 1987; Emami, 1996; Tiwari, 2002). However, this conclusion was derived from crosses between parents with orange and yellow cotyledons. Emami (1996), on the other hand, found that the lentil cotyledons have two types of yellow coloration, viz. bright yellow and dull yellow with brownish tinge. For distinction between the two colour types, the latter was called “brown”. It was also demonstrated that the yellow and the so-called brown cotyledon colours are governed by two independent genes which were given gene symbols *Y* and *B*, respectively (Emami and Sharma, 1996a, b). One more gene was identified during the investigations of Emami (1996) which caused deep green testa in recessive condition and was assigned the gene symbol *Dg* (Sharma and Emami, 2002). The *Dg* gene has epistatic effect on the dominant *Y* and *B* genes in recessive condition and acts at a stage earlier than the metabolic steps effecting the synthesis of the yellow and brown pigments. It was also hypothesized that the product of *Dg* action possibly acts as a common substrate for the action of the genes *Y* and *B*. The recessive condition of these genes, when *Dg* is dominant, results in nonsynthesis of both the pigments simultaneously, and the cotyledons remain light green. The same situation occurs under recessive state of the gene *Dg* when the precursor for the yellow and brown pigments becomes unavailable and the cotyledons acquire deep green colour. These conclusions were made from limited data. The proposed gene interactions and segregation ratios expected in various combinations have been confirmed on the basis of voluminous experimental material involving all possible gene combinations.

Materials and Methods

The study is based on 77 crosses which were made between donors of the three genes in almost all possible combinations. The crosses were made and experimental populations were at the research farm of the Indian Agricultural Research Institute, New Delhi, during winters and at the Wheat Summer Nursery in the Lahaul Valley, Himachal Pradesh, in summer. The seeds were examined for cotyledon colour soon after harvest. The cotyledons were visualized in intact seeds against strong light of ordinary lamp without removing the
seed coat. This technique was standardized by M.K. Emami during his Ph.D. programme (Emami and Sharma, 1996). An instrument for seed examination has been designed subsequently.

Results and Discussion

The main results are summarized in Table 1. The number of seeds analyzed in each class of segregation was in hundreds and thousands, the highest being 65,835 seeds in the category of monogenic segregation for yellow cotyledon colour.

Table 1. Inheritance of cotyledon colour in lentil

<table>
<thead>
<tr>
<th>Contrasting characters</th>
<th>Number of crosses</th>
<th>F1 phenotype</th>
<th>F2 segregation</th>
<th>Ratio tested</th>
<th>( \chi^2 )</th>
<th>P</th>
<th>Heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange × Yellow</td>
<td>39</td>
<td>Orange</td>
<td>65835</td>
<td>21581</td>
<td>3 : 1</td>
<td>4.55</td>
<td>0.05</td>
</tr>
<tr>
<td>Orange × Brown</td>
<td>4</td>
<td>Orange</td>
<td>12053</td>
<td>3944</td>
<td>3 : 1</td>
<td>1.01</td>
<td>0.30</td>
</tr>
<tr>
<td>Yellow × Brown</td>
<td>3</td>
<td>Orange</td>
<td>1929</td>
<td>645</td>
<td>627</td>
<td>229</td>
<td>9 : 3 : 3 : 1</td>
</tr>
<tr>
<td>Yellow × Light green</td>
<td>5</td>
<td>Yellow</td>
<td>8799</td>
<td>2947</td>
<td>3 : 1</td>
<td>0.05</td>
<td>0.83</td>
</tr>
<tr>
<td>Orange × Light green</td>
<td>5</td>
<td>Orange</td>
<td>4536</td>
<td>1455</td>
<td>1506</td>
<td>496</td>
<td>9 : 3 : 3 : 1</td>
</tr>
<tr>
<td>Orange × Dark green</td>
<td>6</td>
<td>Orange</td>
<td>25331</td>
<td>8432</td>
<td>3 : 1</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Yellow × Dark green</td>
<td>8</td>
<td>Orange</td>
<td>10083</td>
<td>4693</td>
<td>9 : 3 : 4</td>
<td>22.24</td>
<td>0.01</td>
</tr>
<tr>
<td>Brown × Dark green</td>
<td>4</td>
<td>Orange</td>
<td>3127</td>
<td>1411</td>
<td>9 : 3 : 4</td>
<td>0.37</td>
<td>0.83</td>
</tr>
<tr>
<td>Light green × Dark green</td>
<td>3</td>
<td>Orange</td>
<td>1943</td>
<td>612</td>
<td>613</td>
<td>210</td>
<td>1183</td>
</tr>
</tbody>
</table>

In agreement with the earlier reports, dominance of the orange colour of cotyledons over yellow as well as brown (i.e. brownish yellow) was confirmed from voluminous data generated in this study with 3 : 1 ratio of segregation for both characters. At the same time, crosses between parents with yellow and brown cotyledons additionally produced F2 classes of double dominant orange and double recessive light green cotyledons in the ratio of 9 orange : 3 yellow : 3 brown : 1 light green. The same digenic F2 ratio with independent assortment was also obtained when genotypes with orange cotyledons were crossed with those having light green cotyledons in five cross combinations (7993 F2 plants). These observations provided strong support to the conclusion that the two dominant genes \( Y \) and \( B \) jointly produce orange cotyledons, while in their recessive homozygous condition the tissue fails to synthesize both the pigments and the cotyledons remain green. It appears that these genes have poor penetrance in recessive condition, as a result, the cotyledons have yellowish tinge and look light green on visual examination.

This also explains the dominance of yellow cotyledon colour over light green observed in a population of 11,746 seeds in the F2 seeds in five different crosses. By the same token, brown colour is expected to be dominant over light green. Although such direct crosses were not made in this study, results supporting this conclusion were obtained when seeds from brown seeded heterozygous F2 plants were advanced to the F3 generation and their progenies segregated in the ratio of 3 brown : 1 light green (data not presented).
The relationship of the gene $Dg$ causing dark green cotyledons in recessive homozygous condition with $Y$ and $B$ was investigated by crossing genotypes having dark green cotyledons with donors of orange, yellow, and brown cotyledons. The orange $\times$ dark green cross gave monogenic segregation in a total population of 33,763 seeds from six crosses even though the orange phenotype itself is a two-gene dominant phenotype. In other words, a single gene in recessive condition ($dg$) blocks the expression of two functional (i.e. dominant) genes $Y$ and $B$. This is what was called recessive epistasis in classical genetics. It also simultaneously proves that the gene $Dg$ belongs to a third locus.

As a consequence of the above situation, the $F_1$ seeds have orange cotyledons when parents with dark cotyledons ($YYBBdgdg$) are crossed with the donors of either yellow ($YYbbDgDg$) or brown ($yyBBDgDg$) cotyledons, and digenic segregation under recessive epistasis in the ratio of 9 : 3 : 4 is obtained.

Table 2. Confirmation of segregation patterns for cotyledon colour in $F_3$ seeds

<table>
<thead>
<tr>
<th>$F_2$ plants (no. Phenotype)</th>
<th>$F_3$ segregation (no. of seeds)</th>
<th>Ratio tested</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orange</td>
<td>Yellow</td>
<td>Brown</td>
<td>Light green</td>
</tr>
<tr>
<td>Orange</td>
<td>234</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Orange</td>
<td>376</td>
<td>0</td>
<td>115</td>
<td>0</td>
</tr>
<tr>
<td>Orange</td>
<td>1770</td>
<td>0</td>
<td>0</td>
<td>600</td>
</tr>
<tr>
<td>Orange</td>
<td>1176</td>
<td>391</td>
<td>0</td>
<td>563</td>
</tr>
<tr>
<td>Orange</td>
<td>449</td>
<td>0</td>
<td>138</td>
<td>0</td>
</tr>
<tr>
<td>Orange</td>
<td>1219</td>
<td>2297</td>
<td>396</td>
<td>126</td>
</tr>
<tr>
<td>Orange</td>
<td>955</td>
<td>287</td>
<td>328</td>
<td>100</td>
</tr>
<tr>
<td>Yellow</td>
<td>0</td>
<td>304</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yellow</td>
<td>0</td>
<td>456</td>
<td>0</td>
<td>152</td>
</tr>
<tr>
<td>Yellow</td>
<td>0</td>
<td>553</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>Brown</td>
<td>0</td>
<td>0</td>
<td>363</td>
<td>137</td>
</tr>
<tr>
<td>Brown</td>
<td>0</td>
<td>0</td>
<td>323</td>
<td>0</td>
</tr>
<tr>
<td>Brown</td>
<td>0</td>
<td>0</td>
<td>411</td>
<td>93</td>
</tr>
<tr>
<td>Light green</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>372</td>
</tr>
</tbody>
</table>

Trigenic segregation with recessive epistasis ($27 : 9 : 9 : 3 : 16$) was observed in the crosses made between parents having light green ($yybbDgDg$) and dark green ($YYBBdgdg$) cotyledons. This scheme of gene interactions was confirmed when the materials were advanced to the $F_3$ generation and progenies of the heterozygous $F_2$ plants were analyzed for segregation (Table 2). Thus, the hypothesis of trigenic control of cotyledon colour in lentil is confirmed from this detailed analysis based on crosses and experimental materials that were not available earlier.
It was interesting to note that two F₂ plants also turned out to be heterozygous for green cotyledons and their seeds segregated for light green and dark green cotyledons in the monogenic ratio of 3 : 1. This creates an impression as if light green is dominant over dark green even though the two phenotypes are a consequence of recessive condition of different genes. In reality, these plants were homozygous recessive for \( y \) and \( b \) and heterozygous for \( D_g \). The epistatic effect of \( dg \) is lost in its homozygous dominant or heterozygous conditions.

The epistatic effect of the gene for dark green cotyledons in homozygous recessive (\( dgdg \)) condition has been interpreted by assuming that the synthesis of the yellow and brown pigments in the cotyledons is catalyzed by the genes \( Y \) and \( B \), respectively, most probably from a common precursor whose synthesis is controlled by \( D_g \) at an earlier step in the chain of metabolic reactions leading to orange cotyledons. The yellow and brown pigments cannot be produced in the absence of the precursor when the gene controlling its synthesis is in nonfunctional (recessive) state (Sharma and Emami (2002)).

**References**


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Change in Content and Chemical Composition of *Hypericum perforatum* L. Oil at Three Harvest time

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**Abstract**

Saint John’s wort (*Hypericum perforatum* L.) is an important medicinal plant that contained a wide range of secondary metabolites such as naphthodianthrones, phloroglucinols and essential oil. The quality of *H. perforatum* was determined by several cultural practices. In this research, essential oil content and composition determined at before flowering, full flowering and fruit set stages. Water distillation was used for essential oil extraction. Essential oil composition was determined by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS). Based upon the results, herb of Saint John’s wort in full flowering stage had higher amount of essential oil than before flowering and fruit set stage. The essential oil contained longifolene, α and γ-eudesmol, spathulenol, bicyclogermacrene, β-caryophyllene, α-cadinol, α-cadinene and β-bisabolene as major constituents. These constituents were affected by harvest time. In this respect longifolene was identified in oil sample before flowering and fruit set stage but unidentified in the oil of full flowering stage. The amount of bicyclogermacrene was the highest (16.93 %) at full flowering stage and decrease sharply at other harvest time. According to the results, the most suitable time for harvesting of *H. perforatum* with respect to essential oil content and composition is full flowering.

**Key Words index**: Essential oil, Harvest time, *Hypericum perforatum* L., St.John’s wort.

**Introduction**

Saint John’s wort (*Hypericum perforatum* L.) is an important medicinal plant that has been used since ancient time due to producing a wide range of secondary metabolites with significant pharmaceutical effects such as wound healing and antidepressive properties. (Bombardelli and Morazzoni, 1995; Cellarova *et al*., 1994; Southwell and Campbell, 1991). Hypericin, a polycyclic aromatic dione is one of the most important secondary metabolites. Other secondary metabolites are essential oils which are accumulated in translucent spheroidal cavities (Cellarova *et al*., 1995; Kreft and Luthar, 1993). Translucent dot are scattered throughout the leaf lamina and extend from abaxile to adaxile epidermis (Cellarova *et al*., 1997; Repcak and Martonfi, 1997). Saint John’s wort belonging to the Chusiaeceae (*Guttiferae*) family occure naturally in Asia minor, Northern Iran and Northern Africa and Europe apart from the arctic regions (Oravec *et al*., 1996). The quality of the essential oil is chiefly determined by its constituents. Although the infra specific chemical differences seem to be genetically determined, but according to several studies, the essential oil are also greatly influenced by environmental and ontogenetical factors (Bernath *et al*., 1991). On the other hand essential oil content and composition of medicinal plant affected by harvest time.

**Material and methods**

This experiment trial was carried out on Mashhad’s University research station. Weather condition and soil characters are shown in Table 1. Seeds of *Hypericum perforatum* L. cultivar “Topas” washed during over night and after air-dried were sown in spring in outdoor bed in 5 mm depth and irrigated regularly (Kordana and Zalecki, 1996). The seedlings of St. John’s Wort has low growth rate (Pluhar and Zelnik, 1994, Seidler and Dabrowska, 1996)
therefore after 6 month seedling height was 25 centimeter. Seedlings were transplanted to
sandy loam soil in the field. The statistical design used was randomized complete block with
three replicates for each treatment. Each plot was 160×125 cm. The seedling planted on row
40 cm apart and seedlings spaced in 25 cm. Hoeing, mechanical weeding and irrigation were
done regularly. In the first year after transplanting, growth rate was very low but in next
spring after satisfying the chilling requirement, the stem initiation took place. Plant harvested
at three different stages such as before flowering (BF), full flowering (FF) and fruit set (FS)
stage and considered as the treatments. Plant materials dried in dark condition at 30±0.5 °C
before analysis.

**Essential oil extraction:** The air- dried aerial parts of plants were subjected to water
distillation for 3 hours using all-glass apparatus (clevenger apparatus) according to methods
recommended by the Hungarian pharmacopoeae(1984). The oils produced were mobile, pale-
yellow in color.

**Identification of compounds:** Many constituents were identified by comparing their Kovats
retention indices according to the literature (Davis,1998; Sandar and Bicchi,1987;
Weyerstahl, et al.,1995). We have also used GC/MASS for component identity confirmation
and further identification.

**Gas Chromatography(GC) analysis:** GC analysis was carried out on a Shimatzu GC-9A fitted
with capillary column (60 m×0.25mm) Silicon DB-1. Carrier gas He. Inlet pressure 3
Kg/cm² split mode. Temperature programmed 50-250 at 4 °C/min, Injector temperature 250
°C, Detector used dual FID temperature 265 °C, Injector volume for all treatment 0.1 µl.

**GC-Mass analysis:** GC-Mass analysis were carried out on Varian 3400 gas chromatograph
fitted with a column such as GC one which was temperature programmed from 40-250 °C at
4 °C/min. Carrier gas He, flow rate 3.7ml/min, Injector temperature 260 °C. The
chromatograph was coupled to Saturn II A mass selection detectore(70eV).

**Results and discussion**

**Essential oil content:** The results indicated that harvest time had significant effect on
essential oil content of *Hypericum perforatum*. As shown in Table 2, second harvest time
(FF) produced more amount of essential oil (0.35 ml/100 g dry weight). The essential oil
content of FF stage was 191 % and 50 % higher than that of BF and FS stages, respectively.

**Essential oil constituents:** Analysis of essential oil of *Hypericum perforatum* showed that
harvest time had significant effect on its constituents. The results of GC and GC /MS were
presented in Table 3. Thirty-nine constituents of the herb oil at BF stage, thirty constituents
of herb oil of FF stage and thirty-seven constituents in herb oil of FS stage were identified by
GC and GC/MS analysis that presented in Table 3. The main constituent of herb oil at BF
stage are longifolen (18.71 %), γ-eudesmol (10.75 %), spathulenol (6.90 %),
bicyclogermacrene (5.51%), β-caryophyllene(4.62%), γ-murolene (4.52%), α-cadinol
(3.20%), farnesol (2.98%), α-eudesmol(2.90%), γ-cadinene(2.17%). The main constituents of
herb oil at FF stage are α-cadinene (27.17%), bicyclogermacrene(16.93 %), spathulenol
(6.95 %), γ-eudesmol(6.52%), α-cadinol(5.70%), α-eudesmol(3.81%), α-
cuprenene(2.44%), β-caryophyllene(2.07%) and finally the main constituents of the herb oil
at FS stage are longifolen(22%), β-bisabolene(9 %), spathulenol(8.45%), glubulol(5.15%),
α-cadinol(4.87 %), α-eudesmol(2.77%), and farnesol(2.23%)(Tables 3). There are striking
difference in the chemical composition of the herb oil at three different harvest time of
H. perforatum L., specially with respect to relative amount of γ-eudesmol, spathulenol, bicyclogerma-crene, β-caryophyllene, α-cadinol and α-eudesmol. Longifolen is present in substantial amount in herb of the plant at BF and FS stage (18.7% and 21.99% respectively) but not detected at FF stage. It was the same as γ-murolene, β-cadinene, β-bisabolol that not detected in FF stage. The amount of bicyclogerma-crene was high (16.93%) at FF stage but decreased sharply during the other harvest times. β-bisabolene and glubulol were present in substantial amount in herb at FS stage but not detected in oil of the herb at BF stage. Some constituents decreased during flowering and fruit set process. For example the highest content of γ-eudesmol (10.75%) detected at BF stage but its content decreased at FF and FS stage (6.52 and 0.72% respectively). This decrease trend was so high for ortho-cymen, geranyl acetate, viridiflorol, as their contents at FS stage were trace. Some constituents such as α-thujen, sabinene, γ-teroinen, α-corae, β-bisabolene, elemol, guaiol, glubulol only were detected at FS stage. This maybe related to fruit set process. According to the results, developmental process such as flowering and fruit set can affect essential oils content and constituent of H. perforatum. Essential oil of Hypericum perforatum L. has commercial interest because it is used in the pharmaceutical industries (Cellarova, et al. 1997). The quality and quantity of active substances of medicinal plants under various ontogenetic stages are different. On the basis of this investigation, the essential oil of Hypericum perforatum L. strongly affected by harvest time and the best time for harvesting of the plants with respect to essential oils yield and its composition is full flowering (FF) stage.

References
8- Hungarian pharmacopoeia, (1984), VII Kiadas , III. kotet , medicinal konyvkiado

Table 1: Climatic and soil characteristics of experimental station

<table>
<thead>
<tr>
<th>Soil analysis</th>
<th>pH</th>
<th>CEC</th>
<th>EC</th>
<th>Organic matter %</th>
<th>N %</th>
<th>P</th>
<th>K</th>
<th>texture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.9</td>
<td>13.8</td>
<td>0.9</td>
<td>0.92</td>
<td>0.05</td>
<td>4.2</td>
<td>305</td>
<td>Sandy loam</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Climatic condition</th>
<th>Altitude</th>
<th>Latitude</th>
<th>Lowest tempt.</th>
<th>Climatic category</th>
<th>Yearly precipitation</th>
<th>Average humidity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1215</td>
<td>35º,43'</td>
<td>-7.6ºC</td>
<td>Semi-dry</td>
<td>242.7 mm</td>
<td>42%</td>
</tr>
</tbody>
</table>

Table 2: Effects of harvest time on fresh and dry herb yield and essential oil content of *Hypericum perforatum* L.

<table>
<thead>
<tr>
<th>Harvest time</th>
<th>Fresh herb yield(T/ha)</th>
<th>Dry herb yield(T/ha)</th>
<th>Essential oil content(ml/100 gr)</th>
<th>Essential oil index in %</th>
<th>Essential oil yield(l/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before flowering</td>
<td>7.5 b</td>
<td>2.0 c</td>
<td>0.12 c</td>
<td>100</td>
<td>2.4</td>
</tr>
<tr>
<td>Full flowering</td>
<td>15.7 a</td>
<td>3.9 b</td>
<td>0.35 a</td>
<td>291</td>
<td>13.8</td>
</tr>
<tr>
<td>Fruit set</td>
<td>18.0 a</td>
<td>4.7 a</td>
<td>0.18 b</td>
<td>150</td>
<td>8.4</td>
</tr>
</tbody>
</table>
In each column, means followed by the same letter are not significantly different by Duncan’s multiple range test, \( P \leq 0.01 \)

<table>
<thead>
<tr>
<th>No</th>
<th>Components</th>
<th>Percent</th>
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<th>Full flowering</th>
<th>Fruit set</th>
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<td>-</td>
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<td>( \beta )-Pinen</td>
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<td>( \alpha )-Humulene</td>
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<td>1.67</td>
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<td>22</td>
<td>( \beta )-Caryophyline</td>
<td>4.62</td>
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<td>23</td>
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<td>-</td>
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A modified CTAB method for isolation of DNA from mint plants (Mentha spp.)

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Abstract
A large number of medicinal and aromatic plants produce secondary metabolites such as alkaloids, flavonoids, phenols, gummy polysaccharides, terpenes and quinines. For genetic improvement of these medicinal plants, it is desirable to use molecular markers for screening of accessions, choosing of parents and selection of progeny. The presence of certain metabolites has been observed to interfere with DNA isolation procedures and downstream reactions such as DNA amplification. It has been thought that the RAPD fingerprinting is sensitive to the quality of the DNA template. Therefore, in the present study, we modified the some essential steps of DNA isolation procedures for mints which yielded high quantity and quality of high molecular weight DNA. This procedure is a modified CTAB method described by Murray and Thompson (1980).

Leaves of different Mentha species were used for DNA isolation. Extraction buffer containing polyvinylpyrrolidone (PVP) was used for better removal of polysaccharides and secondary metabolites. Polysaccharides and secondary metabolites which are abundant in mint leaves are known to be bound by PVP. For purification, DNA was extracted with phenol:chloroform:isoamylalcohol. The DNA samples isolated by the method were used for RAPD fingerprinting of Iranian mints. The results showed successful reproducibility through PCR amplification. This procedure could be applied to some plant species of medicinal and aromatic plants which produced essential oils and secondary metabolites.

Keywords: DNA isolation, RAPD fingerprinting, Medicinal plants, Mints, RAPD fingerprinting, Secondary metabolites

Introduction
A large number of plant species produce secondary metabolites such as alkaloids, phenols, polysaccharides and terpenes that are used in food, confectionary, cosmetics and liquor industries. Molecular marker approaches not only used for breeding of genotypes of medicinal and aromatic plants, but also used for assessing and exploiting the genetic variability of these plants. Developing DNA markers/fingerprints of all the genetic resources of the medicinally and industrially important plants, is a necessity for generating a molecular database as well as to utilize the information in a systematic way.

The presence of certain metabolites can hamper the DNA isolation procedures and reactions such as DNA restriction, amplification and cloning. Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include: (1) degradation of DNA due to endonucleases, consolation of highly viscous polysaccharides, and (2) inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weising et al., 1995). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA (Loomis, 1974, Porebski et al., 1997).
Plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. The chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weising et al., 1995).

Here we described a rapid DNA isolation protocol that can be used for some medicinal and aromatic plants and in particular those taxonomically related with Mentha species. The protocol permitted isolation of DNA from tissues of diverse plant species in fairly good yield, and the isolated DNA proved amenable to PCR amplification.

Materials and Methods

Plant materials

Four species of mints including Mentha spicata, M. piperita, M. longifolia and M. suaveolens were used. Total DNA was isolated from freshly germinated young leaves by following a modification of the CTAB method of Murray and Thampson (1980).

DNA isolation protocol

1. Grind 3 g of fresh tissue to a fine powder using liquid nitrogen and a mortar and pestle.
2. Transfer the powder as fast as possible into 10 ml of pre-warmed 2.5× CTAB extraction buffer [2.5% Cetyl-Trimethyl Ammonium Bromide (CTAB), 1.5 M NaCl, 25 mM Na2EDTA, 100 mM Tris-HCl (pH 8.0), 2% β-mercaptoethanol, 1% polyvinylpyrrolidone] to 50 ml polypropylene tube.
3. Incubate for 60 min at 60°C in a water bath with slow shaking every 10 min.
4. Add 10 ml of chloroform: isoamylalcohol (24:1), and shake slowly for 10 min on a rocker.
5. Spin at 4000 rpm for 15 min at room temperature using a centrifuge rotor.
6. Carefully transfer the upper clear aqueous layer to another 50 ml fresh tube.
7. Repeat step 4 to 6 for more purity of DNA.
8. Transfer the supernatant to a new 50 ml tube and precipitate DNA by adding 2/3 volume of pre-chilled (-20°C) isopropanol.
9. Collect the precipitated DNA by low speed centrifugation (10 min) at room temperature to separate the organic and aqueous phases.
10. Add RNase A to a final concentration of 100 µg/µl and transfer the tube to 37°C for 1 h.
11. Add equal volume of processed phenol to the tube containing DNA and shake for 10 min to form an emulsion and then centrifuged at 3000 rpm for 10 min at room temperature to separate the organic and aqueous phases.
12. Transfer the aqueous phase to a fresh 15 ml tube and add 1/2 volume phenol + 3/4 volume chloroform: isoamylalcohol (24:1) and shake for 10 min and centrifuge at 3000 rpm for 10 min.
13. Transfer the aqueous layer to a fresh 15ml tube and add 1ml chloroform: isoamylalcohol (24:1), mix by rocker for about 10 min and centrifuge at 4000 rpm for 10 min.
14. Transfer the aqueous layer to a fresh 15ml tube, add 1/10 volume sodium acetate and 2.5 volume ethanol -20°C to precipitate DNA.
15. Spool out DNA with glass rod, air dried for 20 min and finally suspended in appropriate volume of TE buffer in a 1.5 ml microfuge tube.

For checking the quality of total genomic DNA, 2 µl of DNA in TE was fractionated by 0.8% agarose gel electrophoresis. DNA concentration in TE was determined spectrophotometrically by measurement of optical density. The ratio between the readings at 260 nm and 280 nm (OD260/OD280) provided an estimate of the purity of nucleic acid. Pure preparation of DNA has OD260/OD280 values of 1.8.

**PCR amplification**

Thirty one arbitrary 10-mer primers were used for PCR amplification of the total genomic DNAs. Polymerase chain reaction was performed based on the protocol of Williams *et al.* (1990), with minor modification. Amplification were carried out in 25 µl of reaction mixture containing 2.5 µl of PCR buffer, 200 µM each of dNTPs, 1.5 mM MgCl₂, 15 ng of the primer, 0.7 unit of Smar Taq DNA polymerase and 25 ng of DNA template. The amplifications were performed using a Eppendorf Mastercycler Gradient thermalcycler. The amplified products were loaded in a 1.2% agarose gel containing ethidium bromide. After electrophoreses, the gel was photographed under UV light.

**Results and Discussions**

The plants that are sources of natural products or bio-active substances also produce large amounts of secondary metabolites and substances of medicinal or industrial importance. Thus while working with a variety of plants it is common to encounter problems arising from the presence of essential oils, polysaccharides, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations. In our experiments we encountered difficulties from the stage of cell lyses to DNA separation in the supernatant and subsequent reactions when following the procedure described by Khanuja *et al.* (1999), and Murray and Thompson (1980). Major problems encountered were low DNA yield or poor PCR amplification reactions.

Khanuja *et al.* 1999 described isolation of DNA from dry and fresh samples of several genera. They evaluated the procedures described by Doyle and Doyle (1987), Dellaporta *et al.* (1983), and Porebski *et al.* (1997). The protocol described by Khanuja *et al.*(1999) used three ml of extraction buffer. In our protocol, we used 10 ml of extraction buffer. Moreover, the procedure also eliminates the necessity of NaCl, and addition of 3M sodium acetate (pH 5.5) for sedimentation of DNA. The problem arising from the presence of high levels of proteins was overcome by using phenol:chloroform:isoamylalcohol. The standard way to remove proteins from nucleic acid solutions is to extract first with phenol:chloroform:isoamylalcohol and then with chloroform. This procedure takes advantage of the fact that de-proteinization is more efficient when two different organic solvents are used instead of one.

The protocol described by Murray and Thompson (1980) involved only addition of β-mercaptoethanol. It has been known that Polysaccharides and secondary metabolites which are abundant in mint leaves are bound by PVP. The addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols from *Mentha* species (Neal Stewart and Via, 1993).

The above protocol invariably achieved good yield of high quality DNA from fresh tissues of above plants. By using the above protocol a fairly high quality DNA was obtained for each plant (Figure 1a). The amount of DNA recovered per gram of plant leaves was sufficiently
high. Table 1 showed yield of DNA isolated from various samples of different species of mint plants using the CTAB procedure modified in this study.

The utility of the isolated DNA for use in PCR amplification for RAPD profiling was demonstrated with several random primers and with DNA preparation of all the plant species tested (Figure1b). The method described here is, therefore, rapid, simple and efficient for the isolation of DNA from plants that possess a wide range of activities that can interfere with DNA extraction and analysis. This procedure can be directly applied to *Mentha* species and closely related genera which produced essential oils and secondary metabolites.

References


Table 1: Yield of DNA isolated from various leaf samples of four *Mentha* species using the CTAB procedure as modified by this study

<table>
<thead>
<tr>
<th>Plant (family)</th>
<th>Number of accession</th>
<th>Range of DNA yield µg/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. longifolia</em></td>
<td>3</td>
<td>100.5 - 173.3</td>
</tr>
<tr>
<td><em>M. piperita</em></td>
<td>4</td>
<td>32.1 - 272.4</td>
</tr>
<tr>
<td><em>M. spicata</em></td>
<td>9</td>
<td>43.7 - 175</td>
</tr>
<tr>
<td><em>M. suaveolens</em></td>
<td>1</td>
<td>135.5</td>
</tr>
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</table>

Figure 1: Isolated DNA from 10 accessions of *Mentha* species electrophoresed by 0.8% agarose gel (Figure 1a), Figure 1b, shows PCR profiles of the DNA samples amplified with primer 5'-CACAggCggA-3' oligo-primer.
DNA-technology in plant production improvement

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Abstract.
Molecular genetics achievements are determinate factor for developing biology in end of 20 and beginning 21 centuries. The progress in creation contemporary biotechnology connected to success in investigation structure and variability DNA. Second “green revolution” based on DNA technology and in vitro tissue culture using and reduce soil erosion and decrease water contamination. Modern biotechnologies save resources and promote to reduce price of agricultural products. Sophisticated systems that simulate internal cell processes were start in pharmacology for drug synthesis and last time find wide application in plant production. In plant production use gene modified plants (GMP), DNA-technology and in vitro tissue culture. GMP in spite of counteraction some organizations, connected with chemical companies and “greens” now occupied about 70 million hectares. In plant breeding and seed production wide distributed molecular markers that modernized and made more efficient traditional breeding and create new approaches for differentiation and identification plant varieties. Tissue culture in plant production permit to get plants from wide crossing and reduce term creation non segregated material in breeding program via dihaploid technique. Molecular markers help to detect molecular genetics polymorphism and are more efficient as morphological and biochemical (proteins) markers. In 90-th of 20 century was created new approach for determinate DNA polymorphism with using polymerase chain reaction (PCR). For short for science developing period PCR-technique find very wide application in plant genetics and breeding and now is powerful tool for solving many problems of plant productions. Different field of PCR apply generate several variant of this technique. Its differ as dominant and co-dominant, monolocus and polyloci, bialleles and polyalleles. Among polyloci are more distributed bialleles, dominant systems like RAPD, ISSR, IRAP, REMAP. RAPD system don’t require information about nucleotides sequence of DNA and can be use without big expenditures for differentiation species and varieties. The data reproducing problem with RAPD generate ISSR variant where known sequences of microsatellites use as a primers. In this case problem with reproducing data is eliminate but polymorphism investigation restricted small fraction of genome. IRAP and REMAP systems involve in study genome variation LTR fragment of retrotransposons. Set of multyloci PCR markers provide genetics mapping and include in plant breeding programs. Monolocus polyalleles co-dominant SSR system attractive for genotype identification, registration source of germplasm and study alleles distribution for loci that connected with economy important traits. SSR markers is very useful for genotype identification. Set of 15-20 pairs primers to hypervariable microsatellites supply unique differentiation of genotypes many agricultural species and can be use as supplement or sometimes instead DUS test for preliminary varieties identification. It is important for marking varieties and protection right of breeders. DNA technology that arouse in end of 20 century as technology of 21 century transform plant production introduce modern sciences achievements into agriculture.

Key Words: DNA-technology, molecular markers, plant production improvement.

Introduction.
Aim of contemporary plant production is supply of sufficient quantity of high performance products for satisfaction human needs and feed for animal. Due to achievements of genetics
and breeding that called “green revolution” in 60-70 years of 20 centuries the productivity of the main agricultural plants was possible considerably to raise.

The new types of high productive plants have demanded an intrusion so-called “intensive technology”. Negative side of this direction it is possible to consider energy output and application of a plenty of fertilizers and herbicides aggravating an ecology. The creation of the most high productive varieties and hybrids on bases hybridization of outstanding genotypes considerably has narrowed down genetic base of many agricultural species.

Development of molecular genetics and gene engineering have created conditions for an intrusion in plant production in essence new technologies. The second “green revolution” is characterized by application of different version of DNA-technology and tissue cultures in vitro technique.

The research of organization and variability of DNA that long time wearing academic nature are basis of high technology in biology that transform notion about plant production.

The biotechnology, in classic comprehension, exists for a long time and is connected to usage of phenomena descending in a living cell, for human needs. The modern biotechnology quite respond this definition, only with addition, that human can significantly changes type of activity living cells. Among major direction of a modern biotechnology it is necessary to distinguish: obtaining transgenic plants, DNA-polymorphism usage, apply tissue and organs culture in vitro.

Introducing inherited information that connected with important agronomical traits from genetic distant species into elite varieties open wide perspectives to improve cultivated plants. In during several decades science overcame way from study influence exogenic DNA to plant to creation genes constructions with isolated important genes. Agricultural plants obtain so relevant properties as resistance to biotic factors, herbicides, viruses etc. Next in turn is creation plants that can synthesis animal proteins and drags. Discussion about negative influence products of genetics modified plants to people health will be very useful for improving quality of this products. To stop historical process of an intrusion of gene-modified organisms into practice of plant production is not real. Gene-engineering technology will not substitute traditional breeding but enrich cultivated species by value in agronomic meaning traits.

In arsenal of contemporary biotechnology the considerable place occupied genetics markers. Three types of markers – morphological, biochemical and molecular are known and use in genetics and breeding of plants. The resovling ability and stability of molecular markers is much higher than others. In last decade of 20 century elaborated technology reveal genetics polymorphism on base of enzyme amplification by polymerizes chain reaction (PCR) of some DNA sequences. Feature of PCR-analysis is:

- an opportunity of work with small of samples
- independence of a stage of development of plants
- an opportunity of work with any tissue of a plant
- automatization of DNA multiplication process
- an opportunity analysis of a plenty samples simultaneously
- high speed getting results
- presence of the computer programs of data processing

The creation of PCR-technology was as a result integration achievements on genome structures investigations, gene engineering, protein chemistry and instrumental methods. One of the first successful PCR system was RAPD that operate random primers. DNA polymorphism cause by mutation in site of primering. In case not association primer with matrix will not detected band of amplicon on electrophoresis. For a rather short time created and wide distributed PCR technology, that include variants that differ in primers design and manner of its inheritance. Polyloci systems include RAPD, ISSR, IRAP, REMAP is useful for establish genetic relations via determination distances between species or genotypes of one specie. In comparative analysis with tens random primers possible to get information about hundreds of loci.
Co-dominant monolocus polyallelic SSR system essentially supplement PCR-analysis. Possibility to detect both alleles in heterozigote permit to analyse “genetic purity” of plant. Determination linkage between SSR-markers and agronomic value traits promote study adaptive meaning some alleles and search distribution some genes and alleles in breeding process.

**Characteristic of some PCR - system.**

For different kind of problems solution were created listed in table 1 variants of PCR-analysis.

**Table 1.**

<table>
<thead>
<tr>
<th>Type of PCR</th>
<th>Type of inherances</th>
<th>Type of primers</th>
<th>Type of polymorphism</th>
<th>Number of amplicons</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSRP</td>
<td>К</td>
<td>Pairs of primers with known sequences, flanked simple repeats of microsatellite</td>
<td>Changing in length (number repeats)</td>
<td>1-2</td>
<td>Tautz, 1989</td>
</tr>
<tr>
<td>AFLP</td>
<td>К*</td>
<td>I stage: primers complimentary to adaptors; II stage: primers content up to 3 b.p. non complementary to adaptors</td>
<td>Point mutation, insertion, deletion</td>
<td>&gt;30</td>
<td>Lin, Kuo; Vos et. al., 1995</td>
</tr>
<tr>
<td>SSAP</td>
<td>Д*</td>
<td>I stage: primers complimentary to adaptors; II stage: one primer contain 1-3 b.p. non complementary to adaptors, second primer complementary to LTR</td>
<td>Point mutation, transposition</td>
<td>&gt;30</td>
<td>Waugh et al., 1997</td>
</tr>
<tr>
<td>RAPD</td>
<td>Д</td>
<td>One, random (decamer) primer</td>
<td>Point mutation, insertion, deletion</td>
<td>10-30</td>
<td>Williams et al., 1990</td>
</tr>
<tr>
<td>AP-PCR</td>
<td>Д</td>
<td>One, random (&gt;10 b.p.) primer</td>
<td>Point mutation, insertion, deletion</td>
<td>10-30</td>
<td>Welsh, McClelland, 1990</td>
</tr>
<tr>
<td>DAF</td>
<td>Д</td>
<td>One, random, (octamer) primer</td>
<td>Point mutation, insertion, deletion</td>
<td>&gt;30</td>
<td>Caetano-Anolles et al., 1994</td>
</tr>
<tr>
<td>ISSR</td>
<td>Д</td>
<td>One, microsatellite repeats with additional 1-3 b.p.</td>
<td>Point mutation, insertion, deletion</td>
<td>10-30</td>
<td>Zietkiewicz et al., 1994</td>
</tr>
<tr>
<td>IRAP</td>
<td>Д</td>
<td>One or two primers complementary to one or two LTR</td>
<td>Point mutation, transposition</td>
<td>10-30</td>
<td>Schulman, 1998</td>
</tr>
<tr>
<td>REMAP</td>
<td>Д</td>
<td>One primer complementary to LTR, second primer - microsatellite repeats</td>
<td>Point mutation, transposition</td>
<td>10-30</td>
<td>Kalendar et al., 1999</td>
</tr>
<tr>
<td>SNP</td>
<td>Д</td>
<td>One RAPD, AP-ПЦР, several on AFLP</td>
<td>Point mutation, insertion, deletion</td>
<td>10-30</td>
<td>D. Wang, 1998, A. Brooks, 1999</td>
</tr>
</tbody>
</table>

К* - co-dominant  
Д** - dominant

Set of PCR markers permit to solve many problems of developing theory and practice of plant breeding and seed production.

**Using IRAP and REMAP PCR for barley varieties differentiation.** The exact characteristic of genetic material is one of the most important aspects of practical genetics. Nowadays the main role in the identifications of varieties belongs still to the morphological, biochemical and other traditional methods. New technologies based on DNA polymorphisms analysis are provided in the last time. These approaches allow to provide on the molecular level analysis of genetic peculiarities of every variety and “pasportisate” the sources of germplasme using molecular markers. The purpose of new system of identification and
registration of genetic resources on the base of DNA-markers data is preservation, using and successful creating of new forms as well as possibility of decision the questions on disputable cultivars authority.

RAPD method was widely used to explore issues of genetic diversity. But RAPD-analysis is not enough reproducible. It was necessary to create stronger criteria of PCR. ISSR-method (Inter-Simple Sequence Repeat) was effective and reproducible but it detected only one microsatellite fraction of DNA (though it has high density within genome).

New PCR-methods: IRAP (Inter-Retrotrasposon Amplified Polymorphism) and REMAP (Retrotrasposon-Microsatellite Amplified Polymorphism) that was proposed by Dr.Kalendar (1999) use one or pair primers to retrotransposons LTR (Long Terminal Repeats) sequence, and one primer to retrotransposon LTR sequence and ISSR-primer, respectively. Retrotransposons as well as microsatellites are widely dispersed through genome. According literature data the perspective direction in the marker assisted selection (MAS) of barley is the using of IRAP- and REMAP-markers.

The following question was investigated in our work: the analysis of intraspecies polymorphisms of investigated barley cultivars of Odessa breeding with the help of IRAP- and REMAP-analysis.

As the material for our investigation 27 varieties of Odessa breeding were used: 20 spring and seven winter species. Polymorphism detection is provided with the help of primers of various barley retrotransposons LTR sequences and ISSR-primers with two PCR-methods: IRAP and REMAP.

![Diagram of 27 barley cultivars](image)

Fig.1   Groupings of 27 barley cultivars on the basis of the genetic distance using data of IRAP- and REMAP-analysis.

*s – spring cultivars; o – winter cultivars.

**2 – two-rows cultivars; 6 – six-rows cultivars.

We have provided the selection of LTR-primers detecting polymorphism on the investigated barley species. The most informative nine variants for IRAP-analysis were...
selected out of the 55 possible combinations of primers. The number of detected amplification products was 143 with 86 polymorphic. The polymorphism level detected in the analysis varied from 50.0 to 73.3 % and average value was 60.2 %.

We have provided the selection of LTR- and ISSR-primers detecting polymorphism on the investigated barley species. The most informative nine variants for REMAP-analysis were selected out of the 122 possible combinations of primers. The number of detected amplification products was 157 with 105 polymorphic. The polymorphism level detected in the analysis varied from 43.8 to 80.0 % and average value was 66.9 %.

The dendrogramm that we get shows intraspecies relation analysis result by program “TREES” is presented by two clusters. The first cluster includes spring barley cultivars, the second – winter ones.

Besides two-rows and six-rows barley cultivars were distributed clearly: 19 two-rows barley cultivars were in the first cluster and one six-rows cultivar Pallidum 107 was separately situated in the first cluster. All six-rows barley cultivars were in the second cluster.

DNA-MARKERS FOR HETEROSIS PROGNOSIS. Maize breeders continuously face the choice of inbred lines to be tested. Many studies have discussed the interest of distance indexes between parental lines in order to achieve this choice, in particularly the use of molecular-genetic markers. Several authors have found a positive relationship genetic distances between parents and F1 yield if the large number of marker loci were considered in study.

The aim of this studying is to develop the DNA-marker system for heterosis prognosis in maize simple hybrids. The following tasks were decided: differentiation of inbred lines by ISSR- and SSR-markers; count of genetic distances between lines from ISSR-analysis data; correlation analysis of genetic distances between paternal lines with the index of hybrids productivity; associations search of allele composition of lines microsatellite loci with the level of heterozygoity of hybrids.

Plant materials: 15 maize inbred lines from early group of ripeness: Od7, Od17, Od18, Od24, Od139, Od141, Dk2/165, Oh43, X5753, R221, PLS61, W401; Ekc15, BAM97, Ok109. Use PCR with 10 ISSR-primers and 20 SSR-primer pair; electrophoresis in agarose and polyacrylamide gels; UPGMA-analysis.

The indexes of genetic distances (D) between lines varied from 0.033 to 0.538. According to D-indexes lines are parted on four groups: I - D<0.2; II – 0.2<D<0.3; III – 0.3<D<0.4; IV - D>0.4. On the basis of lines distributing we assumed that hybrids from crossings of I group lines would be to have the low level of heterosis, and hybrids got from more genetically divergence paternal pair from the II-IV groups, would have more high level of heterosis, increasing as far as increasing of genetic distances between initial lines.

In 2002 lines crossing is conducted and information about seed harvest of paternal lines and proper hybrids is got in 2003. True heterosis (H) was defined on the seed harvest. The mean value H made 108 %.

The groupment of initial lines, carried out by the D value, was compared with distributing of heterosis level. The mean D-value of I group lines is 0.091, II group is 0.245, III group is 0.353, IV groups is 0.456. The mean H-value of hybrids got from crossing of I, II, III and IV groups lines made 66, 78, 144 and 143 % accordingly. The correlation coefficients r of genetic distances between lines and heterosis level of their hybrids are following: 0.60; 0.46, 0.61 (it is reliable at P=0.05) and 0.41 (it is not reliable) for lines the I, II, III and IV lines groups accordingly. As far as the increase of genetic distances indexes between initial lines there is reliable growth of heterosis level of the proper hybrids. On the basis of low indexes of genetic distances between lines "excluding" of the closely related crossings is possible, and the got high indexes of genetic distances will allow to forecast the receipt of hybrids with high level heterosis.

PCR-analysis of 20 microsatelite loci allowed to get the sets of alleles, individual for every genotype. Gomozygote state of explored loci is for all lines. Information about lines genotyping is represented in the table 1. Amplified alleles were encoded "1", "2", "n" from
small molecular weight to high. The comparative analysis of line SSR-allele composition and average heterosis at the hybrids got from crossing of this line with all other showed reliable correlation (P=0.05) on nine microsatellite loci (the values are selected by bold type in table 1). Groupment of lines on three groups on the level of heterosis of the proper hybrids (I group is heterosis to 100 %; II group – 100-150 %; III group – higher 150 %) were associated with SSR-allele composition and heterosis level, that gave possibility to write down the model genetic formulas of line X: A1B2C1K1M1R1S1U1 and line Y: A2B2C2D1E1I1K2M2O2S2T2U2, probably possessing a different combination ability (letter means microsatellite loci, down index – allele number, got on this locus for this genotype). If at SSR-genotyping some line allele composition on loci A, B, C, K, M, R, S, U will be the same as at model line X (thus allele composition on other loci can be any), it is possible to assume that for this line low combination ability is characteristic. Like, at the coincidence of allele composition of some line with such line Y, this line will possess high combination ability.

Table 2. Comparison of maize hybrid heterosis level with parental lines SSR-allele composition

<table>
<thead>
<tr>
<th>Line</th>
<th>OJ139</th>
<th>OK109</th>
<th>OJ24</th>
<th>W401</th>
<th>BAM9</th>
<th>X5753</th>
<th>OB43</th>
<th>EKC15</th>
<th>DK21</th>
<th>OJ141</th>
<th>OJ18</th>
<th>PL651</th>
<th>OJ17</th>
<th>OJ7</th>
<th>R221</th>
<th>Average heterosis, %</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>phi001 (A)</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>3</td>
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<td>3</td>
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<td>3</td>
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<td></td>
</tr>
<tr>
<td>phi064 (B)</td>
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<td>2</td>
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<td>3</td>
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<td>3</td>
<td>3</td>
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<td>4</td>
<td>4</td>
<td>3</td>
<td>0.767604</td>
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<td>2</td>
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<td>phi093 (J)</td>
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<td>phi008 (K)</td>
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<td>phi085 (L)</td>
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<td>phi091 (P)</td>
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<td>2</td>
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<td></td>
</tr>
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</table>

Lines SSR-genotyping information was used for determination of allele composition of corresponding hybrids, taking into account that inheritance of allele is carried out on the Mendel law (in each locus the hybrid F1 must contain one allele from maternal line, other – from paternal line). Hybrids groupment carried out on heterosis level (I group is heterosis to 50 %; II – 50-100 %; III – 100-150 %; IV – higher 150 %) was compared with allele.
composition of microsatellite loci. Reliable correlations (P=0,05) between the heterosis level and allele composition of 7-11 SSR-loci for different groups of hybrids were got. For every group of hybrids most often meet allele combinations were choused (table 3). Correlation between heterosis level and allele combinations (reliable coefficients at P=0,05 are by bold type) were counted. Coefficient of correlation of heterosis level and total allele set for 20 SSR-loci were 0,773 (reliable at P=0,05). Combination of allele composition and loci composition, characteristic for the certain group of hybrids, was defined. The unique combination of alleles and loci in the supposed hybrid, calculated by initial lines genotyping, will allow to deliver it into a group with the certain heterosis level, that will eliminate the ineffective crossings.

<table>
<thead>
<tr>
<th>Heterosis level</th>
<th>Most frequent combinations of SSR-alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 50 %</td>
<td>phi001 phi004 phi01127 phi083 phi029 phi0047 phi0076 phi0053 phi0085 phi0129 phi0070 phi0051 phi1001 phi1016 phi1015 phi0107 phi0401 phi0971</td>
</tr>
<tr>
<td>50 – 100 %</td>
<td>phi001 phi004 phi01127 phi083 phi029 phi0047 phi0076 phi0053 phi0085 phi0129 phi0070 phi0051 phi1001 phi1016 phi1015 phi0107 phi0401 phi0971</td>
</tr>
<tr>
<td>100 – 150%</td>
<td>phi001 phi004 phi01127 phi083 phi029 phi0047 phi0076 phi0053 phi0085 phi0129 phi0070 phi0051 phi1001 phi1016 phi1015 phi0107 phi0401 phi0971</td>
</tr>
<tr>
<td>&gt;150 %</td>
<td>phi001 phi004 phi01127 phi083 phi029 phi0047 phi0076 phi0053 phi0085 phi0129 phi0070 phi0051 phi1001 phi1016 phi1015 phi0107 phi0401 phi0971</td>
</tr>
</tbody>
</table>

Correlation coefficients

0.796782 0.816199 0.824107 0.77139 0.17419 0.774597 0.7746 0.774597 0.7746 0.774597 0.7746 0.774597 0.7746 0.774597 0.7746 0.774597 0.7746 0.774597 0.7746

Thus, molecular-genetic identification and degree estimation of initial material matter very much in practical heterosis breeding. Empiric selection of most productive genotypes from thousands hybrid combinations results in the enormous volume of uninforming test crossings, in considerable expenses of time and facilities and not always provides desirable result. Introduction in breeding practice of DNA-technology for initial material estimation allows to conduct more purposefully pick up paternal pairs for high heterosis hybrids.

**DNA-markers of QTL in maize breeding.** The discovery of molecular markers opens a new area for quantitative genetics and selection of complex trait. Until now quantitative genetics could develops genetics laws for quantitative traits without identifying the genes involved. By tagging the genome, “invisible” genes become “visible”, which makes it possible to identify the genotype. As a result genotypic values can be derived from the genotype at marker loci. It becomes also possible to use knowledge about the recombination between loci for accumulating favorable genes in the same genotype. We use of QTL DNA-markers to increase the accuracy of the prediction of genotypic values.

The researches aim: developing of DNA-markers of maize quantitative trait loci and creation on their basis of new technologies for increase of efficiency selection process.

Genetics material – inbred lines ГК26, Mo17 and segregate population (ГК 26 x Mo17)F2; generations F3 – F6. Measure of 18 most important of quantitative traits was carry out. The PCR-methods of the DNA-analysis such as SSR, ISSR and RAPD were used for tagging of quantitative trait loci.

The variability of quantitative traits and heritability of DNA polymorphic fragments were analysed for detection linkage association. DNA-markers of QTL, which essential influence on development of yield, morphology and biochemical traits were developed. The tagging
ability of QTL DNA-markers was confirmed in two generations to growth in contrast environment.

Markers cannot explain all the variation of a complex traits. It follows that the best way of predicting genotypic values is a combination of two types of information: about the phenotype and about the markers. In our study we use two technology: prediction and selection based on both phenotype and markers as a version of marker-assisted breeding. The key concepts to predicting and selection is that information one marker will be not correct. It was confirm also by experimentally in our study. Therefore we must be used complete information of a few markers loci. As a result “marker test systems” was proposed for increase of correct analysis of quantitative trait variability and probability of prediction. Requirements to formation of markers test system and informative markers criteria for increase of DNA-predict and markers selection models efficiency are proved.

Technology of DNA-prediction (fig.2).

1. Forming of effective marker test system: a) the number of markers for test system – if population size near 200 individuals shell be used for combine 4 dominant markers, 3 codominant with dominant markers and only 2 codominant markers; b) by “framework” will be best codominant locus; c) combine dominant markers in same test system and dominant with codominant markers will be need for identical marker allele state; d) consequent of marker locus segregating for theoretically.

![Fig. 2. A. Combine marker data.](image)

The basis of technology of DNA-predicting is regression analysis of connection between combine marker data for specific trait and its phenotype variability. Such analysis we began since F2. After genotyping F3 by regression coefficient predicted of develop level for study traits until sowing. Planting will be only individual which has characters interesting for breeders. Capacity of our technology was checked in experimental models of DNA-predicting. The level of reliable prediction consists 40-83% while satisfactory level of model experiment consider 30%.

Technology of QTL DNA-markers based selection (fig.3).

Additional criteria for forming of effective marker test system: a) the best allele state is only homozygote, because heterozygote loci in next generation will be segregated and expressed also negative QTL-information; b) linkage marker loci increase accuracy of selection. The selection begins since F3 after check of markers reliable in two generations. Effective of this step depend on intensive index: \( S = C_0 - xC_{ms} \), where \( C_0 \) – one cycle selection population, \( C_{ms} \) – population of marker selection. \( C_{ms} \) reproductions and getting of population \( C_1 \).

Effective of this step depend on move index: \( R = xC_1 - xC_0 \). On based index \( R \) calculate genetic effect of selection: \( \Delta G = R/x \times 100\% \).
Fig. 3. Relative effect of QTL DNA markers based selection comparative with traditional breeding.

Capacity of this technology was checked in experimental models of QTL DNA-markers based selection. The genetic effect of selection consists 9.1-18.7% while for traditional breeding method only 4.1 at one selection cycle.

Creating technology have proposed for predicting of individual genotypic value of quantitative traits, genetics improve of populations, developing of synthetics specially on complex agronomic traits as origin material for new inbred lines and following heterosis selection.

**SSR-markers using for varieties identification.**

By definition of the International convention on protection of new varieties of plants (Geneva, 1997) "variety" designates as a group of plants within the framework of lowest of known botanical taxons which in independence of that, completely it satisfies whether or not to conditions of submission on a legal protection:

- Can be characterized by a degree of display of traits which realizations of the given genotype or a combination of genotypes grow out.
- Can distinct from any other groups of plants by a degree of display even one of traits,

It can be considered as a unit from the point of view of its fitness for reproduction in a constant kind of plants of a variety. Thus, the variety represents genotype or a combination of genotypes which to the traits differ from other groups of plants. The genotype in modern representation of molecular genetics is characterized by a set of alleles which differ at each organism or group of organisms from others. The existing system of identification of the variety, accepted in UPOV, defines variety of agricultural plants by three criteria, the so-called DUS-test: distinguishability, uniformity, stability. The DUS-test is based on phenotypes characteristics, for the description which cultivation of plants till a full maturity is necessary. Climatic conditions can vary depending on of years that will be reflected in display of phenotypes characteristics. DUS test demands the big expenses of time and results of it frequently are not unequivocal.

Essential progress in development of molecular genetics, especially, different systems of molecular markers, allows to add DUS-test and reduce terms of definition of a variety and permit to use for identification insignificant amount of a vegetative material at any stage onthogenesis. The special attention involves a variant of the PCR-analysis-SSRP. SSR-markers - codominante, one locus, polyalleles. SSR represent simple repeating sequences of hypervariable microsatellites. Microsatellites are distributed on all genome, cover all chromosomes. Variability is connected to quantity of repeating elements in different genotypes. Use of SSR-markers of 15-20 loci allows is unique to differentiate varieties of wheat, barley, corn, etc. cultures. Practice shows, that at use of sufficient number of markers all researched varieties it is possible to differentiate and define genetic distances between them. The criterion of uniformity too is determined in this system of markers. If the variety will consist of several genotypes, they differ by allelic condition of microsatellite loci. If the variety represents a combination of genotypes each of them is designated as a set different alleles. The system of molecular markers is capable to fix a genotype of a hybrid. Advantage of molecular markers is that for definition of a genotype there is no necessity to raise a plant before full maturing. For allocation DNA enough several cells of a sheet, a root, a stalk or a
With the help of molecular markers there is an opportunity to create bank of the varieties registered in country and to carry out the control over a direction of selection process.

The received sizes of fragments of amplification of each microsatellite locus, i.e. the sizes alleles in pairs nucleotides (bp)., write down as the formula in which the letter of the latin alphabet designates a code of a SSR-locus, the bottom index the size of alleles in bp. In case of a homozygous condition of a locus one is underlined only one allele.

Example of formula of genotype of wheat variety Albatros:
\[ A_{79}B_{186}C_{147}D_{191}E_{204}N_{192}G_{134,140} \]
\[ H_{121}I_{178}J_{131}K_{163}L_{192,200}M_{125}N_{215} \]

Example of formula of genotype of wheat variety Odesskaya 51:
\[ A_{77}B_{186,192}C_{141,149}D_{191,193}E_{204,208}F_{192}G_{140,142}H_{119,121}I_{170,178}J_{101,119}K_{129,163}L_{200}M_{125,133}N_{215,218} \]
Application of gamma radiation for pistachios and Dates disinfestation and packaging materials

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Abstract
Different gamma radiation doses on different developmental stages of two important stored-pests of pistachios and dates, Indian meal moth Plodia interpunctella (Hbn) and Saw-toothed weevil Oryzaephilus surinamensis (L) was investigated. Results showed that 0.7 kGy of gamma radiation can control all developmental stages of the two insects and 0.35 kGy cause sterility in both of them. Eight kinds of polymers were used as packaging materials. They were as follows: BOPP, OPP/PE, PP/PP, PP/PE, PE/PS, PET, PET/PVDC and PVC. Results showed that PET, PET/PVDC and OPP/PE were resistant to Plodia and all the packagings except PE/PS and BOPP were resistant to Oryzaephilus. However, on the basis of the observations, PET80µ was the best in terms of elasticity, sealing properties, resistance to insects and keeping the product fresh. In other experiment, polymers irradiated with 1.0 kGy and comparing with non-irradiated ones, results showed no significant difference among them. In other experiment, the interactions of 0.5 and 1.0 kGy radiation and different polymers on pistachios and dates characteristics such as texture, taste, odour and colour was investigated during one year. The results showed that between irradiated treatments and controls no significant difference were observed, however, irradiated dates with 0.5 and 1.0 kGy had better quality in terms of rancidity and granulated sugar on it. Nutritional ingredients were analyzed in terms of protein, fat and sugar in pistachios and dates after packaging with PET80µ as the best packaging material and irradiation with the obtained disinfestations dose 0.7 kGy and compared with controls, no significant difference was observed. Pilot scale experiment under laboratory condition for ensuring of results was done and the result showed that after 6 times sampling it was not any insect contamination and packaging puncture in irradiated ones compared to control with 45% contamination in dates and 15.5% in pistachios.

Irradiation facility for the project was provided by gamma-cell of 4100 Ci/Co60 source at a dose rate of 0.64 Gy/s installed at NRCAM, Karaj, Iran.

Key words: Irradiation- stored pests- nuts- disinfestation- packaging

Introduction:
Iran is the first producer of pistachios and dates in the world with total production of 205,000 and 900,000 tonnes, respectively [7]. Two important stored-pests of pistachios and dates are Indian meal moth Plodia interpunctella (Hbn) and Saw-toothed weevil Oryzaephilus surinamensis (L) [4]. For the time being, one of the most effective and prevalent methods of disinfestations is using fumigants. Some fumigants have undesirable effects on products especially on fruits and vegetables, also reducing germination of seeds. It should not be forgotten the risk of explosion of fumigants either. Methyl bromide is one of the prevalent fumigants. On the basis of Montreal protocol, developed countries till 2005 and developing countries until 2015, have to eliminate using it, because of its ozone depleting properties [5]. In the US in 1984, Ethylene dibromide was banned in food and agricultural commodities, because of carcinogenic properties, suspected [9,10]. Japan, Germany and other European countries, thereafter, did that. Irradiation as an alternative to fumigants, for spice...
disinfestations and dried stored products was debated. In 1998, 70,000 tonnes of irradiated products was sold worldwide [5].

Materials and Methods

2.1. Determining lethal and sterile dose for *P. interpunctella* and *O. surinamensis*

*P. interpunctella* was reared on artificial diet containing: 2 parts wheat + 1 part oats + 1 part bran + ½ part dry yeast powder + Glycerol 15 ml/100 gram dry ingredient. The diet was decontaminated at 60°C temperature for 6 hours. For egg collection, the adults were placed in the funnel and it was put on the radiology film for 24 hours. The eggs laid, were transferred onto the diet and were kept in incubator at 28±2°C and 55±5% Rh in plastic jars for rearing. The dimension of plastic jars was 17×17×25 cm.

*O. surinamensis* was reared on ground wheat. The diet was decontaminated at 60°C for 6 hours. The adults were placed on the diet for rearing and were kept in plastic jars and were kept in incubator at 29 ± 2 ºC and 60 ± 5 % Rh. The dimension of jars was 17×17×25 cm. When the population of insects grew sufficient, the four developmental stages of each insect (egg, larva, pupa and adult) were irradiated. The lethal and sterile radiation doses for each developmental stages ranged between 0.05-0.8 kGy for *P. interpunctella* and 0.05-0.7 kGy for *O. surinamensis*, respectively. The results in both species are based on 50 samples of every developmental stages replicated four times. In both species, for egg and pupa, the criterion was the ability of creating next stage, namely, larvae and adults. In the case of larvae and adults, sampling was done at specific intervals and the results were recorded.

2.2. Evaluation locally available packaging materials for pistachios and dates

Eight types of packaging materials were obtained including: BOPP 30 micron, PVC28, PP/PP50, OPP/PE70, PP/PS80, PET80, PE/PS80, PET/PVDC80 for testing on: Determining resistance of packaging material to a minimum dose of 1.0 kGy. This test was replicated four times. Each replication was one square meter. Irradiated and non-irradiated films were sent to "Poushineh Plastic Co." for quality analysis and comparison covering items including: thickness, density, haze, transmittance, opacity, gloss, sealing strength, seal initiation temperature, treatment type, treatment intensity, tensile, elongation, elasticity modulus, static friction coefficient, shrinkage and charge decaying rate.

Second part of the research was related to surveying the suitability of different above mentioned packaging materials against *P. interpunctella* and *O. surinamensis* penetration/reinfestation. This part has been done with contaminating the inner part of the packaging material with 10 first instars larvae, sealing the packaging and 100 gram decontaminated pistachio in outer part of the packaging and were kept in large plastic jars to survey ability of insect penetration and puncturing towards the product. This test has been done in opposite way and the durability and resistance of different packaging materials were determined by the number of holes created by insects. Each test was replicated four times and the samples were kept under the same controlled rearing condition of *P. interpunctella* and *O. surinamensis*. Sampling was conducted weekly for 2 months.

2.3. Evaluation sensory quality test for irradiated and non-irradiated pistachios and dates

This test was conducted to evaluate the sensory quality of irradiated pistachios and dates with the doses of 0.5 and 1.0 kGy and compared with the non-irradiated one in terms of texture, taste, odour and colour. The samples were packed with the above mentioned packaging materials for pistachios in two kinds of sealing as conventional and vacuum, but for dates only conventional sealing (because date is damaged by vacuum). The test was replicated six times for each packaging material and every sample contained of 200 gram of pistachios and dates. The samples were stored in 10±1°C and 45±5% Rh. Six referees with high degree of
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precision tested the samples at two months interval during 12 months and their evaluation were recorded as "very bad, bad, moderate, good and very good".

2.4. Analysis of irradiated and non-irradiated pistachios and dates in terms of ingredients

By using suitable packaging material, pistachios and dates irradiated with the obtained dose and together with non-irradiated were sent to different institute for determination of in gradients such as: protein, sugar, fat.

2.5. Pilot scale experiment under laboratory condition for ensuring of results

This experiment was conducted using the best packaging material and irradiated with the obtained control dose for *P. interpunctella* and *O. surinamensis* to confirm the result. The experiment was replicated six times and packed with the best packaging material (PET 80 micron). Each packaging type contained 1 kg of pistachios and dates which were infested by 10 factors of each developmental stages of two mentioned insects which include, eggs, 4th instars larvae, pupae and adults and irradiated with obtained control doses for all developmental stages and compared with non-irradiated. All the samples were stored at 25±2°C and 60±5% Rh. Samples examined monthly for insect disinestation and packaging resistance to insect punctures during 6 months.

Results

3.1. Determining lethal and sterile doses for *P. interpunctella* and *O. surinamensis*

There was no emergence of eggs irradiated with 0.05 kGy for both species. The egg stages sterile dose was in the range of 0.035-0.05 kGy. The *Plodia* larval stage was controlled with 0.65 kGy within 14 days and 0.2 kGy within 35 days after irradiation. The *Oryzaephilus* larval stage had 100% mortality with the dose of 0.09 kGy within 7 days. The sterile dose for controlling larval stages of two species is in the range of 0.03-0.09 kGy. In the case of pupa, 0.35-0.65 kGy for *Plodia* and 0.6-0.7 kGy for *Oryzaephilus* are sufficient doses for controlling. The sterile dose for pupal stage of *Plodia* was 0.35 kGy and in this stage the emerged adults were malformed and could not lay eggs and the sterile dose for controlling *Oryzaephilus* was 0.085 kGy. In the adult stage of *Plodia* 100% mortality was obtained with the dose of 0.5-0.65 kGy comparing 0.115-0.145 kGy for *Oryzaephilus* during 14 days after irradiation. The sterile dose for adult stage of *Plodia* 0.35 kGy and for *Oryzaephilus* was 0.085 kGy.

<table>
<thead>
<tr>
<th>Dose (kGy)</th>
<th>Egg</th>
<th>Larva</th>
<th>Pupa</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>O</td>
<td>P</td>
<td>O</td>
</tr>
<tr>
<td>Lethal</td>
<td>0.05 - 0.2</td>
<td>0.025 - 0.05</td>
<td>0.2 - 0.65</td>
<td>0.09 - 0.12</td>
</tr>
<tr>
<td>Sterile</td>
<td>0.05</td>
<td>0.035</td>
<td>0.09</td>
<td>0.03</td>
</tr>
</tbody>
</table>

3.2. Determining the sensory quality of irradiated pistachios and dates subjected to a minimum dose of 0.5 and 1.0 kGy

Based on the results obtained, there was no significant difference between irradiated and non-irradiated pistachios. In the case of dates, some samplings indicated that 0.5 and 1.0 kGy treatments had better quality in comparison with control. It was indicated that packaging materials can be effective on preserving dates but it makes no difference on pistachios. According to statistical calculations in Duncan test with 0.05 error, it was concluded that there was no significant difference among packaging materials in all treatments (control, 0.5 and 1.0 kGy) considering texture, taste, smell and colour of pistachios and dates. However some of them have more preference because they were more desirable in terms of resistance
to punctures made by insects, sealing properties and etc. Therefore, the sequence of preference are listed as table 2.

Table 2: Comparing the first four packaging material that are accepted and the second four that are rejected

<table>
<thead>
<tr>
<th>Pistachios (Conventional sealing)</th>
<th>Pistachios (Vacuum sealing)</th>
<th>Dates (Conventional sealing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>PET</td>
<td>PET</td>
</tr>
<tr>
<td>OPP/PE</td>
<td>OPP/PE</td>
<td>OPP/PE</td>
</tr>
<tr>
<td>PET/PVDC</td>
<td>PET/PVDC</td>
<td>PET/PVDC</td>
</tr>
<tr>
<td>PP/PP</td>
<td>PP/PP</td>
<td>PP/PP</td>
</tr>
<tr>
<td>PE/PS</td>
<td>PE/PS</td>
<td>PE/PS</td>
</tr>
<tr>
<td>PP/PS</td>
<td>PP/PS</td>
<td>BOPP</td>
</tr>
<tr>
<td>BOPP</td>
<td></td>
<td>PVC</td>
</tr>
</tbody>
</table>

3.3. Evaluation of the locally available packaging materials with regard to their resistance to irradiation with a minimum dose of 1.0 kGy as well as their suitability for packaging dried fruits and nuts for irradiation

1) According to different tests that have been done in "Poushineh Plastic Co", the results show that there is no significant difference between irradiated packaging material with the dose of 1.0 kGy and control, only in the irradiated samples of PET/PVDC, static friction coefficient and kinetic friction coefficient have been improved compared to non-irradiated which is very important in application of packaging materials.

2) The effectiveness of different packaging materials was determined from the number of punctures created by insects and the percentage of infestation in two sides of the material. According to the results (table4), PE/PS, PP/PS, PVC, PP/PP and BOPP were not resistant to P.interpunctella larvae but PET, PET/PVDC and OPP/PE were resistant. According to the results (table5), all the packaging materials except PP/PS and BOPP are resistant to O.surinamensis larvae.

Table 3: Comparing different packaging materials in term of physical and mechanical characteristics

<table>
<thead>
<tr>
<th></th>
<th>PET</th>
<th>OPP/PE</th>
<th>PET/PVDC</th>
<th>PP/PP</th>
<th>PE/PS</th>
<th>PP/PS</th>
<th>BOPP</th>
<th>PVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haze</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Transmittance</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Gloss</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Opacity</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Sealing strength</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Sealing initiation temp.</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Treatment type</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Treatment intensity</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Tensile</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Elongation</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Elasticity modulus</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Static friction coefficient</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Kinetic friction coefficient</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
Shrinkage & 4 & 4 & 2 & 5 & 5 & 4 & 4 & 1 \\
Charge decaying rate & 3 & 4 & 2 & 3 & 5 & 4 & 4 & 1 \\
SD±M & 4.46±0.62 & 4.0±0.0 & 3.73±1.16 & 3.8±0.67 & 3.2±1.08 & 2.46±0.74 & 3.93±0.26 & 2.33±1.29 \\

1: very bad  2: bad  3: moderate  4: good  5: very good  SD±M: standard deviation±mean

Table 4: Number of punctures and damage percentage produced by *P. interpunctella* larvae

<table>
<thead>
<tr>
<th>Packaging material</th>
<th>OPP/PE</th>
<th>PP/PP</th>
<th>PET/ PVDC</th>
<th>PET</th>
<th>PE/PS</th>
<th>PP/PS</th>
<th>PVC</th>
<th>BOPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside packaging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pistachio</td>
<td>4</td>
<td>3</td>
<td>50%</td>
<td>1</td>
<td>3</td>
<td>50%</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Inside packaging</td>
<td>4</td>
<td>1</td>
<td></td>
<td>3</td>
<td>50%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Number of punctures and damage percentage produced by *O. surinamensis* larvae

<table>
<thead>
<tr>
<th>Packaging material</th>
<th>OPP/PE</th>
<th>PP/PP</th>
<th>PET/ PVDC</th>
<th>PET</th>
<th>PE/PS</th>
<th>PP/PS</th>
<th>PVC</th>
<th>BOPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside packaging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pistachio</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inside packaging</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In regard to the results in Tables 2, 3, 4, 5 and the results of sensory quality, PET, OPP/PE and PET/PVDC are the best packaging materials among all that tested

3.4. Conducting ingredient analysis of irradiated and non-irradiated pistachios and dates.

As it is shown in table 6, there is no important observable difference between ingredient of irradiated and non-irradiated pistachios and dates.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Pistachios</th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>8.7</td>
<td>0.45</td>
</tr>
<tr>
<td>Serine</td>
<td>9.97</td>
<td>0.37</td>
</tr>
<tr>
<td>Phenyl-alanine</td>
<td>7.16</td>
<td>0.22</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.44</td>
<td>0.92</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.31</td>
<td>0.23</td>
</tr>
<tr>
<td>Isolusine</td>
<td>6.04</td>
<td>0.13</td>
</tr>
<tr>
<td>Valine</td>
<td>9.68</td>
<td>0.26</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.14</td>
<td>0.15</td>
</tr>
<tr>
<td>Proline</td>
<td>8.77</td>
<td>1.24</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.48</td>
<td>1.12</td>
</tr>
<tr>
<td>Treonine</td>
<td>6.3</td>
<td>0.33</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.7</td>
<td>0.52</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Methionine</td>
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<tr>
<td>Aspartic-acid</td>
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</tr>
<tr>
<td>Glutamic-acid</td>
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</tr>
<tr>
<td>Fat</td>
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<td>-</td>
</tr>
<tr>
<td>Sugar</td>
<td>-</td>
<td>0.721</td>
</tr>
</tbody>
</table>

3.5. Conducting pilot scale experiments to confirm the result under laboratory condition

The results shows that According to 6 times sampling it was not any insect contamination and packaging puncture in irradiated ones compared to control with 45% contamination in dates and 15.5% in pistachios.

Conclusions
- *Plodia interpunctella* and *Oryzaephilus surinamensis* are the most destructive pests of dried fruit and nuts. Radiation sensivity of the two insects was determined at different
developmental stages. The results show that all developmental stages are controlled by the lethal dose of 0.7 kGy and the sterile dose of 0.35 kGy. Consequently, the dose of 0.7 kGy recommended for disinfestations of pistachios and dates [2,4,6,11,12,13].

- Determination of the degree of destruction studies shows that the dose of preventing destruction of pistachios by *O.surinamensis* is 0.075 kGy and for dates 0.065 kGy and the dose of preventing destruction of pistachios by *P.interpunctella* is 0.4 5 kGy and for dates 0.55 kGy.

- In terms of selecting suitable packaging materials due to irradiation and resistance to influence of *P.interpunctella* and *O.surinamensis* the result shows that between three favourable packaging materials PET, OPP/PE and PET/PVDC, (PET80 micron) in view of tensile, sealing, puncture resistance and keeping product fresh is more favourable than the two others.

- In sensory quality evaluation the result shows that there is no significant difference between irradiated and non-irradiated pistachios however irradiation especially with 1 kGy dose could be effective for preserving pistachios and in some cases it is more delicious and fresher .In case of dates radiation decrease crystallization of sugar significantly. Both products show less fungal and insect contamination when irradiated compared to control. Last not the least both products are preserved better and fresher in PET80 micron packaging material.

- Study high quality of pistachios and dates with using the appropriate packaging material (PET80 micron) and irradiation by the determined infestation dose (0.7kGy) compared to control, shows no important observable difference. Consequently Irradiation will not produce difference in pistachios and dates in gradient with the obtained dose.

**Acknowledgement**

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IN VITRO PLANT HEAVY METAL ION RESISTANCE
-A NEW APPROACH OF CELL SELECTION

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Abstract
In vitro cell culture is a suitable method for the detection mechanisms of stress tolerance as well as for the selection of resistant genotypes. Exclusive model systems with toxic concentrations of heavy-metal ions is the alternative approach to obtain and describe cell variants, resistant to number of abiotic stresses. A lot of cell lines of tobacco, sunflower resistant to barium, some oxyanions have been selected. Ba-tolerant cell lines of sunflower and tobacco were distinguished by salt tolerance: several cell lines of sunflower challenge 10 g/l and cell variants of tobacco even 20 g/l of sea water salts. V- or W- resistant cell lines of tobacco grew on selective media without reduced forms of nitrogen and molybdenum and with the addition of toxic concentration of anions. V-resistant regenerants show some new distinct traits of nitrate reductase.

Key words: abiotic stress, heavy metal ions, cell selection.

Introduction
Abiotic stresses are considered to be the most severe limiting factors of crop production. Rash people activity extremely deteriorates the environment via secondary salinity, xenobiotics and heavy metal pollutions. Therefore the importance of the selection of plant varieties with the higher level of stress tolerance can be scarcely exaggerated.

For many years a plant cell culture was established in selection programmes for stress tolerance. Chaleff and Ray (1984) reported about herbicide-resistant mutant from tobacco cell cultures. Conner and Meredith (1985) obtained fertile plants from 40 homozygous diploid cell cultures of Nicotiana plumbaginifolia, resistant to 600 mM of aluminum ions. All of them transmitted the resistance to their progeny. A number of experimental approaches vindicate that in vitro cell selection is a means of increasing plant salt tolerance (Dowton,1984, Larkin and Scowcroft,1981, Raghava Ram and Nabors,1985).

Salinity is a versatile stress factor and affects the whole plant organism in various manners. Therefore it is necessary to remember about cell mechanisms of salt tolerance and how those mechanisms relate to salt tolerance of the entire plant. It is often shown that cell lines selected from wild type cells tolerated higher NaCl concentrations than original ones, but there are solitary facts about an increased salt tolerance being expressed in regenerated plants. Thus some authors put a question “Why does in vitro cell selection not improve the plant salt tolerance ?“ (Dracup,1993). It is assumed that failure of cell selection is due to poor methodology and reluctance to search for mechanisms and test hypotheses. Salt tolerance is correlated with drought tolerance, and salt tolerant organisms are found to be more drought tolerant than susceptible species. At the same time, usually, the toxicity of heavy-metal ions and stress affect of the salinity are investigated separately.

It is known, that divalent metal cations are structurally very similar. Some of the heavy-metal ions are physiologically toxic analogs of biologically important ions. A number of them are widely investigated, some of them are absolutely unexplored. Barium-ions is a such "dark horse".
There are scant publication about barium-ion characteristics. It is known, that these ions reduce chloroplast number and plant photosynthesis. Barium cations essentially affect K⁺ ion efflux (Fan et al., 1999). But on the other hand K⁺/Na⁺ intracellular exchange depends on external ion concentrations (salinity level). Some authors associated the better growth of tolerant callus at high NaCl with low internal Na⁺ concentration and high K⁺/Na⁺ ratio (Gibbs et al., 1989).

Thus, we have formulated a working hypothesis about possibility of in vitro selection for salt tolerance by means of experimental system with barium-ions. To take their inhibitory effect the most heavy- metal ions have to enter to the cell. The two possible types of uptake systems for heavy- metal ions are determined (Nies, 1999). The first type is fast unspecific, constitutively expressed systems, that used various substrates. The second one is slower, has a higher substrate specificity. It is activated in cell at starvation or at special metabolic situation. Thus, the resistance to heavy- metal ions can directly depend on ion transporters, metabolic pathways or accumulated compartments. As a consequence, it appears to be the probability for the selection the plant forms with new parameters (even new types) of cell compounds. Abiotic stresses influence upon plant organism and inhibit its enzyme systems. Nitrate reductase (NR, EC 1.6.6.1) is a basic enzyme of nitrate assimilation in plants. NR from higher plants is homodimeric enzyme, containing a molybdenum cofactor at the catalytic site. A number of factors, such as salinity, mineral nutrition, heavy-metal ions strongly affect NR-activity and structure. NaCl –salinities from the 10 g/l concentration cause the dissociation of molybdenum cofactor from apoprotein. Tungsten (at the form of tungstate-anion) can substitute molybdenum in the cofactor structure and it results in enzyme inactivation (Deng et al., 1989, Heimer et al., 1969). Another oxyanion the vanadate ion, inhibited purified NR from Chlorella vulgaris in a manner similar to that of HCN: inactive complex with the reduced enzyme was formed (Ramadoss, 1979). That is why, we have proposed the second working hypothesis: the stable NRA under stress conditions is one the possible ways towards plant stress resistance. In vitro plant resistans to heavy-metal ions via cell selection is a suitable approach to achieve the result.

Materials and methods
For our investigation we use Nicotiana tabacum L. cv. Samsun and Helianthus annuus L cv. Peredovick. Initial calli were induced from sterile plants of both genotypes. Tobacco and sunflower calli were cultivated on B5 Gamborg nutritional medium (Gamborg et al, 1968). A model selective system were devised.

Ba- ion toxicity. To nutritional medium B5 Gamborg we added toxic for plant cell cultivars of wild type concentrations of Ba²⁺ ions. There were such minimal concentration that inhibited the cell growth. The cell growth and viability were not restored under normal condition. The model salinity was established via addition sea water salts or Na₂SO₄ to nutritional medium. Stress concentrations of both salts were 20 g/l for tobacco cultivars and 10 g/l for sunflower ones.

Oxyanions toxicity. For the investigation of oxyanions toxicity sodium tungstate or sodium vanadate were added to B5 modified medium. There were such modifications: the lack of the molybdenum and the solely nitrate form of nitrogen. NR-activity was determined in V-resistant cell lines of tobacco and regenerated plant as described by Antipov et al, (1998). We investigated tobacco and sunflower cell tissues. From plant callus of wild type suspension cultures in a liquid B₅ medium were established. Cell suspensions were grown in 250 ml Erlenmeyer flasks (25 ml of suspension per flask) on gyratory shaker at 100 rpm. 0,5 ml aliquots of cell suspensions were plated in Petri dishes between two layers of solid selective medium with heavy metal ions. Petri dishes were incubated at 25°C and regularly checked for expanding colonies. The primary colonies were transferred to the same media, containing
initial concentrations of ions. Every 30 days tolerant calli were transferred to fresh selective medium (from the third passage to the media with increasing level of heavy-metal ions).

Such principal scheme of the in vitro selection we always use for isolation and posterior investigation any resistant plant cell line.

Results and Discussion

Ba- resistance.

Sunflower tolerant cell lines retained their viability even under stress pressure of 4 mM Ba- ions. As for tobacco cultivars-during a year there were selected cell lines, resistant to 8.5 mM barium cations. From the third passage Ba-resistant cell lines were cultivated under salinity. Ba-resistant cell variants of sunflower maintained growth on media, containing 10 g/l sea water salts or Na2SO4; tobacco cell cultures developed on media with higher (20g/l) salinity levels. Growth is expressed as relative growth (Δm) and calculated as: Δm = (m₁ - m₀)/m₀, where m₀ – is the initial fresh weight of the callus and m₁ – is the fresh weight at the end of the culture passage.

We observed the increasing callus biomass within any passage. But can the cellular growth under such levels of stress pressure be the reliable index of a salt resistance?

Hitherto there were obtained a lot of salt tolerant cell lines from various plant species. There is a significant genetic variability in the levels of their tolerance. Tobacco variants are considered to be tolerant, if cells sustained even 1% of NaCl in nutritional media (Dix and Street,1975,Hasegava et al.,1980). The response of the alfalfa selected callus tissues was similar to halophytic plant, i.e. active growth was observed in the presence of 1% of NaCl (Groughan et al.,1978). Salt tolerance in cell suspension cultures of the halophyte Kosteletzkya virginica was demonstrated in the medium, containing maximum 255mM (15g/l) of NaCl (Blits et al., 1993). Tolerance in the salt-selected lines was built up by stepped increasing the concentration of NaCl ( Dix and Street 1975,Nabors et al.,1975,Vieira dos Santos2000). Vieira dos Santos et al obtained cell lines of sunflower tolerant to high salinity (200 and 300mM NaCl). But the initial concentration of NaCl in their selection procedure was only 50 mM (2.95 g/l). Wild type callus died, when it was exposed directly to 200 or 300 mM of NaCl.

According to those publications (and our previous investigations) test concentrations of stress substances in our experiments (sea water salts or Na2SO4) are absolutely toxic for unselected cultivars. Thus, the recording of the growth of Ba-resistant tobacco and sunflower cell lines under any type of salinity is a reliable index of their resistance.

It is marked that salt-selected cultivars (calli) often demonstrate a higher resistance than regenerated plants (Dracup 1993). We have obtained regenerants from some resistant cell lines of tobacco. We investigated shoots under stress conditions. Shoots grew and rooted in vitro on selective medium, containing 20 g/l of sea water salts within the passage (35 days) and demonstrated the same level of a desirable feature as cell cultivars.

Regenerants from Ba-resistant cell lines to tobacco were cultivated in vitro more that two years under normal conditions. From their leaf segments the secondary callus was initiated. This secondary callus was transferred to selective media, containing 20 g/l of seawater salts or Na2SO4. Callus tissue retain such level of resistance and was growing under salinity.

We observe the persistence of definite feature at the system: selected cell cultivar – plant-cell cultivar (secondary). We conducted the procedure of cell selection only for barium – ion resistance. We have solved the first problem. The second one- is the investigation of this phenomenon.

Oxyanions resistance

It is evident, that stable NR with a high enzyme activity is a guarantee of the stress resistance of any plant. In vitro selection is the appropriate method to achieve the result. But there were
obtained and studied in details a lot nitrate reductase – deficient (NR) mutant lines. There are two types of NR cell lines. The first (nia-type) is defective in the NR apoprotein, while the second (cnx-type) is the cofactor – mutant form. Selective systems with tungsten or vanadium oxyanions are a model approach to obtain cultivars with alterations of NR. Singh et al have obtained a tungsten – resistant mutant of *Nostoc muscorum* (Singh et al., 1993). This cyanobacterium demonstrated V-dependent nitrate assimilation.

We have devised *in vitro* selective systems with toxic for wild type cells concentrations either tungstate or vanadate anions without reduced forms of nitrogen, without molybdenum. Under such severe pressure we have isolated W – and V – resistant cell cultivars of tobacco and soybean. Tolerant cells grew on media with selection anion and showed cross resistance. On the one hand, our experimentally determined concentrations of oxyanions inhibit the growth of control. On the other hand we mark the increasing of callus biomass. Both events, which occurred simultaneously, indicate for the nitrate assimilation under stress conditions.

The assay of nitrate reductase in some V – resistant cell lines of tobacco and regenerant from these cultivars was carried out. Variants were characterized by negative enzyme activity. All possible modification of NR measurement (addition methyl viologen, benzyl viologen, NADH, NADPH) did not restore the nitrate reductase activity. The procedure of reconstitution of molybdenum cofactor by reaction with extract of *Neurospora crassa* nit-1 mutant demonstrated the lack of Moco. Those facts indicate the absence of normal nitrate reductase.

Antipov et al., reported about molybdenum – free nitrate reductases from vanadate – reducing anaerobic bacterium *Pseudomonas isachenkovi* (Antipov et al.,1998)). Authors underlined, that isolated enzymes differ significantly from all known nitrate reductases. Earlier we have shown, that salt – resistant mutant of tobacco obtained via cell selection, had halophytic type of NR (L’vov et al.,1992). We have already obtained some information about new phenomenon. Investigations will continue.

No doubt, that the problem of heavy – metal toxicity is one of the most complicated. For our investigations we use means of *in vitro* culture and cell selection. Model systems with toxic concentrations of heavy–metal ions is a new approach of *in vitro* selection for stress resistance. It creates a real possibility of appearance of stress resistant plants.

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THE ROLE OF ENERGY METABOLISM IN HETEROSIS FORMATION IN PLANTS

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Abstract

A comprehensive study of genome structural and functional organization and the processes of hereditary information realization in productivity formation in F1 heterotic hybrids is particularly urgent. Combination of molecular-genetic, physiological and biochemical methods enables genome marking, estimation of morphogenetic nature of complicated agronomic and biological properties of plants, as well as revealing of heterosis interaction mechanisms in the systems determining these traits. In this context attention should be paid to the study of multienzymatic complexes, which occupy a key position in cell metabolism. The system, promising for the heterosis mechanism study, is, in our opinion, bioenergy. Effectiveness of biosynthetic processes, adaptation to environmental factor exposure and productivity of agricultural crops depend on their potential energy and perfection of photosynthesis and respiratory metabolism regulation. A comparative analysis of genetic variability was carried out, and a degree of selective pressure on the level of bioenergy equivalents controlling the functioning activity of biosynthetic processes in lines, cultivars and F1 hybrids in maize, tomato and fiber flax was evaluated. The high-heterosis hybrids were shown to exhibit more intensive energy-generating system functioning in comparison with the low-heterosis forms. The revealed differences in the values of reducing and energy equivalents in plant tissues of heterotic and non-heterotic hybrids have defined individual energy-generating system contribution and rearrangement distinctions of their functioning at heterosis. Depending on intergenic and nucleocytoplasmic interaction pattern of genetic material, favorable conditions for a discrete effectiveness rise of the cell energy-generating system may arise in hybrids F1. High balance of bioenergy and growth processes finally results in manifestation of heterosis for productivity. The parental form disbalance between energy generation and growth is due to increased energy consumption for regenerating and maintaining cell structures in an active state. Changes in the regulatory control of bioenergy systems in these genotypes have a negative effect on the rate of biomass accumulation in plant organs and final productivity.

Key Words: bioenergy, fiber flax, heterosis, maize, tomato, lupine.

Introduction

An advanced level of knowledge on heterosis mechanisms indicates that a single factor cannot be a universal cause of hybrid vigor appearance (Milborrow, 1998; Tsaftaris, Kafka, 1998; Birchler et al., 2003; Titok, 2004). With the development of molecular-genetic models of heterosis, among hypotheses of “biochemical enrichment”, “balanced metabolism”, “hybrid protein”, it was established that genetic hypotheses advanced earlier only postulate and don’t explain superiority of hybrid organisms. The hypothesis proposed by K. Mather and resolved by N. Turbin was a successful attempt to found a general theory of heterosis devoid of disadvantages of the theories on dominance and overdominance (Khotyljova et al., 1991). One of the main points of the hypothesis consists in that superiority of hybrids over parents manifests itself not only in growth,
which is a particular case of heterosis manifestation, but also in diverse traits – biochemical, physiological and morphological which are the basis of heterosis formation for productivity. Proceeding from the conception on the determinative role of bioenergy metabolism in viability of plant organism, the goal of the study consisted in a comparative analysis and generalization of an ample experimental material for revealing the most important components of energy metabolism in productivity formation at heterosis.

**Materials and Methods**

Dormant seed, etiolated and green seedlings, plant leaves and stems at different ontogenesis stages in cultivars and F₁ hybrids of maize, yellow lupine, tomato and fiber flax (*Zea mays* L., *Lupinus luteus* L., *Lycopersicon esculentum* Mill., *Linum usitatissimum* L. spp. *usitatissimum*) were taken as a research material.

For solving the stated problem, a multitest physiological and biochemical approach, based on estimating bioenergy characteristics, growth distinctions in parent forms, differing in general combining ability and yield, and F₁ hybrids with unequal heterosis rate for productivity, was used (Khotyljova et al., 1991). When determining activity of bioenergy processes, the following data were used: 1) integral parameters of energy metabolism (IPEM) – content and ratio of nicotinamide coenzymes (NC) and adenyl nucleotides (AN) – \( \text{NAD}^+ \), \( \text{NADH}_2 \), \( \text{NADP}^+ \), \( \text{NADPH}_2 \), AMP, ADP, ATP; 2) activity of a multienzymatic complex of key enzymes – Glucose-6-phosphate (G-6-PD), 6-Phosphofructokinase (6-PFK), Cytochrome-C-oxidase (Cyt-c-oxidase) – individual links of cell respiratory metabolism (pentose phosphate pathway, glycolysis and electron-transport chain of mitochondria respectively). Distinctions of growth processes were assessed by the values of morphological traits and physiological parameters (plant height, number and weight of seeds, biomass dynamics and quantitative parameters of organ growth in seedlings and whole plants, content of pigments, etc.). The contents of AN and NC were determined by the method of high-effective ion-pair HPLC (Titok et al., 1995), activity of enzymes and quantity of pigments were estimated by the spectrometric method (Titok et al., 1994; 1998) and electrophoresis was performed after (Yurenkova et al., 2001). The values of redox charges were calculated by the equations: adenylate energy charge (AEC) = \( \frac{[\text{ATP}] + 0.5 [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \) (Atkinson, Walton, 1967); catabolic reduction charge (CRC) = \( \frac{[\text{NADH}_2]}{[\text{NAD}^+] + [\text{NADH}_2]} \) (Andersen, von Meyenburg, 1977); anabolic reduction charge (ARC) = \( \frac{[\text{NADPH}_2]}{[\text{NADP}^+] + [\text{NADPH}_2]} \) (Andersen, von Meyenburg, 1977); redox charge (RC) = \( \frac{[\text{NADH}_2] + [\text{NADPH}_2]}{([\text{NAD}^+] + [\text{NADPH}_2]) + ([\text{NADP}^+] + [\text{NADPH}_2])} \) (Quebedeaux, 1981).

The results obtained were statistically processed using methods of variance, correlation and regression analyses. Significance of genotypic differences was evaluated by the least significant difference at \( P \leq 0.05 \) (HCP₀₅).

**Results and Discussion**

Knowledge on mechanisms of energy generation in cell and on the ways of its application allows revealing of main points in different metabolic system interaction representing individual stages of intracellular energy metabolism. Interrelation between metabolic cycles occurs by intermediate metabolites, which are substrates simultaneously for several enzymatic reactions; by involvement of enzymes capable of metabolizing several substrates; via “shuttle” mechanisms by using common substrates. The principle of interaction between generation processes and energy application in the form of AN and NC is of important biological value because a flexible system of energy
distribution over different metabolic pathways is created (Raymond et al., 1987; Stasolla et al. 2003).

Investigations pursued at the stage of etiolated maize seedlings have shown that heterotic F_1 hybrids are characterized by more intensive functioning of energy-generating systems as compared to nonheterotic ones. Organ specificity and age dynamics of bioenergy status of seedlings were revealed (Khotyljova et al., 1987). A comparative analysis of maize lines and hybrids has established excess in biomass and the content of ATP and Σ AN over the best parent in etiolated seedlings of those hybrid combinations where the line exhibiting high general combining ability was a maternal form (Titok et al., 1989). Heterotic hybrids, characterized by a high content of AN and NC already at early ontogenesis stages of plants, are shown to be formed by crossing this line with nonrelated lines. When this line is crossed with the related one, nonheterotic hybrids with a low level of macroergic compounds and reducing equivalents are formed (Titok et al., 1989; Khotyljova, Titok, 1994). There is apparently a “weak point” in related lines in the system of energy metabolism. It may be assumed by the distinctions of changes in the system of energy metabolism in etiolated seedlings of these lines that inbred depression affects in the first place the system of oxidative phosphorylation (Khotyljova et al., 1987). Inhibition of the process activity in the line with a high general combining ability is less pronounced than in the related line with a low combining ability that may be due to a higher rate of its inbred depression. Restriction on the rate of a biochemical substrate flow along metabolic pathways seems to be lifted in hybrids produced by crossing the line exhibiting a high general combining ability with unrelated lines owing to a complementary combination of regulatory alleles that leads to heterosis. A comparative study of IPEM in seedling organs in heterotic and nonheterotic forms has allowed characterization of the individual energy-generating processes and rearrangement peculiarities of their functioning at heterosis in the F_1 as well as has revealed their depression in the F_2 hybrids (Khotyljova, Titok, 1994). The results obtained point to compound polygenic of energy traits that seems to be caused by different contribution of individual links of the energy-generating system in etiolated seedlings to the total energy pool (Titok, 1989). Since heterosis for productivity is provided by effective functioning of energy-generating system, it is believed that favorable conditions for discrete increase in the efficiency of one or several components of the energy-generating systems may arise depending on the pattern of intergenic and nucleocytoplasmic interactions. It ensures high balance of bioenergy and growth processes and results in heterosis (Khotyljova, Titok, 1994; Titok et al., 1991). Regularities, observed in analyzing variation in morphophysiological and bioenergy parameters at the initial ontogenesis stages of hybrid and parental maize forms, were revealed in studying cultivars and F_1 hybrids of yellow lupine and fiber flax at the stage of etiolated seedlings (Khotyljova et al., 1993; Titok, Yurenkova, 2000; Titok et al., 1994; 1998a; 2000).

The results of coordinated proceeding of the basic vital processes of plants (photosynthesis, respiration, etc.) are integrated at yield formation. Comparison between parental and hybrid forms of tomato and fiber flax has revealed a heterotic effect for the content of total chlorophyll (Chl) in leaves and stems which was caused by simultaneous increase in Chl a and Chl b (Titok et al., 1994). It should be noted that the F_1 hybrids, along with a high content of photosynthetic pigments, had a dense mesophyll of what specific surface leaf density is indicative. This creates optimum conditions for intensive CO_2 assimilation; as a result, leaf apparatus capacity and biomass accumulation with plant increase (Titok et al., 1994). The analysis on functioning individual links of
respiratory metabolism in green tissue of hybrid and parental forms of tomato and fiber flax under study has shown that the activity of pentose phosphate pathway and glycolysis is inhibited to a different extent as compared to that in etiolated seedlings. Limitation of these cycles is more pronounced in hybrids than in parents in which their relatively high activity may result from insufficient quantity NADPH₂ and ATP in cytoplasm (Titok et al., 2004). In parental forms the values of AEC and NC reduction charges (RC and CRC) are more characteristic of the energy-accumulating system: high rate of energy metabolism, resulting in disturbance of regulatory control of energy-generating and energy-consuming processes, was noted in them that exerts a negative effect on accumulation of organic matter and productivity formation (Titok et al., 1995). Higher Cyt-C-oxidase activity was found in green leaves of heterotic hybrids as against the parents, i.e. besides photosynthesis, mitochondrial respiration makes a substantial contribution to formation of macroergic compounds (Titok et al., 1998b). It follows from this that heterotic F₁ hybrids exhibit more vigorous bioenergy potential creating favorable metabolic conditions in cell for functioning growth-synthetic processes, which favor formation of high productivity.

The study on bioenergy metabolism in fiber flax cultivars and hybrids during ontogenesis has shown that rise in the content of ATP and NAHPH₂ in leaves of the investigated genotypes to the “flowering” stage points to an increase in the reaction activity of the energy-generating system and a decrease in the AN- and NC-levels to the “green ripeness” phase indicates a drop in metabolic load in plant tissues (Titok et al., 1998b; Titok, 2001). Significant excess of energy parameter values in heterotic hybrids over parents during ontogenesis points to an increased activity of energy-generating processes. The AEC value is shown to be able to regulate the intensity of plant growth and development processes via a mediated effect on functioning activity of different metabolic systems (Titok, 2002). Heterotic hybrids exceeded parents and non-heterotic forms in this parameter at all the ontogenesis stages that points to their higher energy provision. As for the AEC value, non-heterotic hybrids occupied an intermediate position between parents, except for the “flowering” stage at which they exceeded them. Relatively low ARC values in parental cultivars at the “fir-tree” stage caused by a decreased functioning rate of the energy-generating system seem to result from the influence of the so-called “limiting factors” which can limit interaction and activity of individual links in energy metabolism. Probably, the heterotic effect for productivity in the hybrid combinations under study is the result of compensatory variation in the activity of biochemical reactions as against the parents. It may be accounted for by the fact that fiber flax cultivars, having “weak points” in different links of bioenergy metabolism, were used for crossing. Accordingly, realization of the genetic potential in the F₁ hybrids provides high tolerance to stress factors and extensive adaptive properties under variable environmental conditions owing to balanced metabolism, i.e. results in heterosis.

Investigations pursued by the isoenzymatic analysis of fiber flax cultivars and hybrids have revealed polymorphism of enzymatic systems (6-Phosphogluconate Dehydrogenase, Glutamate Dehydrogenase, Shikimate Dehydrogenase, Acid Phosphatase, Aspartate Aminotransferase, Cytochrom-C-oxidase) (Titok et al., 1998b; Yurenkova, 2003). Comparison between isoenzymatic spectra of hybrids produced by crossing cultivars, not differing between themselves in the number, electrophoretic mobility and staining rate of enzyme isoforms, has shown that their enzymograms were similar to parents. However, the activity of their manifestation in gel was much higher in hybrids. This may be due to the fact that the activity of loci determining enzyme
synthesis of both parents is equal in hybrids, i.e. additive inheritance is observed. Such hybrids are characterized by a large quantity of enzyme protein thanks to what a change in conditions of proceeding different metabolic reactions in plant tissue cells occur. Isoenzymatic spectra of hybrids, produced by cultivars, whose zymograms differed in the number or electrophoretic mobility of enzyme fractions, included isoenzymes of both parents, i.e. the hybrids spectrum consisted of a greater number of isoforms. The results indicate that the hybrids display greater biochemical diversity owing to the presence of heterozygous alleles in their genome than parental cultivars. Formation of enzyme isoforms in hybrids differing in kinetic and regulatory properties may result in an increase of multienzymatic complex assembly, activation of alternative pathways of individual links in metabolism and in realization of heterosis advantage.

Application of multitest physiological and biochemical analysis in combined investigations of quantitative traits of growth, development and productivity in various agricultural crops allowed formulation of basic points in bioenergy conception of heterosis:

✓ Heterotic effect is realized only under provision of a cell with macroergic and reducing equivalents. High levels of macroergic compounds in the form of ADP, ATP as well as reducing equivalents – NADH₂, NADPH₂ in the cell remove competition between the processes directed at new formation and sustention of organism structure elements, subcellular components, growth and productivity of plants. Co-ordination of generation and consumption rates of bioenergy equivalents in the heterotic F₁-hybrids is an example for balance of the energy metabolism system.

✓ Owing to heterozygosity greater biochemical diversity (increase of variants in multienzymatic complex assembly, extension of conditions of metabolic reaction proceeding, etc.) is formed in hybrid organism than in parents. The presence of heretozygous alleles in hybrid genome assumes emergence of different enzyme forms differing in kinetic and regulatory properties. Formation of dynamic multienzymatic associations in cell of hybrid plant forms and a rise in the functioning activity of bioenergy pathways promote realization of heterosis advantage.

✓ Hybrid vigor is caused by the change in regulation mechanisms of energy metabolism functioning thanks to the presence of different alleles in heterozygote favouring lifting of strict restriction to the activity of growth processes. A combined effect of a great number of such changes results in a rise in the rate of growth processes and in duration of ontogenesis stages in geterotic hybrids of agriculturals crops.

✓ In heterozygous organisms balance and complementary combination of regulatory alleles of different quality determining functioning of individual links in energy metabolism lift rate restriction of the biochemical substrate flow along metabolic pathways that finally results in heterosis. A pool size of bioenergy equivalents is a sensitive activity indicator of biosynthetic processes, which are correlated to yield of the heterotic F₁ hybrids.

✓ In homozygous lines the charge values of nicotinamide coenzymes and adenyl nucleotides present the metabolic situation typical for the energy-accumulating system that is caused by isolation of energy generation and growth processes. Disturbance in regulatory control of the energy-generating and energy-consuming systems in inbred forms indicates high intensity of energy metabolism that affects negatively the rate of organic matter accumulation and ultimate productivity.

✓ Heterosis is caused by the bioenergy balance occurring in a heterozygous state on removal of genetic blocking owing to compensatory action of parental form genomes carrying segregated loci of “weak point” in energy metabolism. A positive
complementation between photosynthesis and different links of respiratory metabolism favors a rise in stability and efficiency of energy exchange that leads to heterosis. Based on the results obtained one may conclude that application of bioenergy markers as criteria for estimating the starting breeding material for heterosis allows selection of genotypes exhibiting physiological and biochemical complementation that provides efficiency of the total metabolism and, as a results, high productivity of heterotic F₁ hybrids.

Acknowledgements
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References


Usage of seeds and branches enzymatic pattern studies for genetic diversity of Juniper habitats in Binalood and Hezarmasjed locations.

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Abstract
Juniper (*Juniperus excelsa*) is one of the most important species that have a wide distribution in many regions of Iran. Thousands hectares of northern, southern and western regions of Iran has been covered by Juniper forest ecosystems. Since one of the most important factors in ecosystem management is genetic diversity of plants, it has been tried to use two enzyme systems (peroxidase and amylase) for genetic diversity determination (in order to rehabilitation program application). Binalood is one of the Juniper forests in the northeastern of Iran. Its elevation is 1800-2123 m and its surface is more than 1000 hectares. The second sampling region is Hezarmasjed with 2000 m elevation and more than 6600 hectares surface. In these regions three different phenotypes could be observed. 30 individuals were sampled within both regions and different phenotypes. Enzyme studies were done by (Polyacrylamide) PAGE method and samples were taken from seeds and branches respectively.

Results showed that seed enzymatic pattern is more suitable than the branches pattern. Moreover by means of this method affected phenotype of Juniper by environmental habitat conditions and human activities could be separated from genotypes (ecotypes). Finally results showed that genetic diversity in Binalood was lower than the Hezarmasjed region. Therefore it needs protective programs for rehabilitation goals.

Introduction
Juniper (*Juniperus excelsa*) is one of the most important species that have been distributed among different mountains of Iran. Based on the many environmental limitations per its habitats, and so presence of different destructive parameters like as human activities, some of its habitats have been destroyed and need to rehabilitation programs.

Khorasan is the highest provinces of Iran with two huge Juniper habitats as the Binalood and Hezarmasjed Mountains. This study was done at both of them. Binalood is one of the Juniper forests in the northeastern of Iran. Its elevation is 1800-2123 m and its surface is more than 1000 hectares. The second sampling region is Hezarmasjed with 2000 m elevation and more than 6600 hectares surface. Range of elevation is 1700-2400 m at Binalood and 1000-2800 m at Hezarmasjed region. The mean precipitation is about 400 mm at the Hezarmasjed but at Binalood is lower than it (1). Based on the overgrazing and human activities, Binalood has been destroyed and the canopy cover of this habitat was scattered. But Layen is opposite of it, with an intensive canopy cover and high rate biodiversity that has been excluded as a reserved area from 1997. Therefore there are two different models for rehabilitation management. But in order to normal rehabilitation, should be charactered genetic diversity situation of every habitat.
**Material and methods**
There are different methods for genetic diversity determination. At first all of the phenotypes were studied and classified to three different classes as follows:

I = The best form with a symmetrical crown, single trunk and without any physiological weakness.

II = The medium form with non symmetrical crown and double trunk

III = The Third form with a distributed break crown and several trunk similar to shrubs forms

Then the most suitable intensity of canopy cover were chosen for sampling, then it has been sampled a plot per every regions contain a .5 hectare surface. Then 10 individuals within every classes of phenotype were chosen and were sampled from seeds and branches. Samples were kept at the +4°C and were transferred to laboratory. The samples were extracted with a 1/3 relation for branches and 3 cc of extraction buffer for every 42 seeds. Qualitative studies were done by PAGE (Polyacrylamide Gel Electrophoresis) method (2). Peroxidase and amylase were chosen as two most important enzymes in all of the plant metabolism process and most suitable enzyme for genetic classification.

**Results**
Enzyme patterns of seeds (Figures 1 & 2) and branches (Figures 3 & 4) showed that seed pattern is more suitable than the branches. Moreover seed patterns of peroxidase showed that Hezarmasjed genotype has some differences with Binalood. In addition seed patterns of amylase showed that the II class & I have a similar genotype and III is related to others. Branches patterns of peroxidase showed similar results to seed patterns but amylase patterns for branches was not.

![Figure 1- Seed peroxidase pattern within Hezarmasjed and Binalood regions](image-url)
Figure 2- Seed amylase pattern within Hezarmasjed and Binalood regions

Figure 3- Branches peroxidase pattern within Hezarmasjed and Binalood regions

Figure 4- Branches amylase pattern within Hezarmasjed and Binalood regions
Discussion
As previously mentioned, rehabilitation program must be designed on the basis of different parameters and genetic diversity is one of the most important of them. Based on the results, genetic diversity in the Binalood region is limited than the Hezarmasjed, may be regard to regressive process of succession. Moreover seed amylase and branches peroxidase patterns show that III phenotype is different with two others in this region. Therefore this phenotype must be considered as one of the genetic classes of seed sources for rehabilitation program (3). Moreover in Hezarmasjed region all of the three phenotypes have different classes based on the amylase patterns and all of them must have a suitable portion for reforestation program.
In addition results showed that seed enzyme patterns are a valuable indicator for genetic assessment of Juniper habitats.

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Genetic improvement of soybean with use complexes of compensating genes

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Abstract

It is well known, that genomes of alive organisms, including self pollinated plants in phylogenesis are exposed to influence of different mutagen factors. The semilethal mutations which are essentially not touching reproductive functions of an organism, due to horizontal transfer of genes and meiotic recombination promote accumulation of a complex dominant and co dominant genes (CCG) compensating negative effect of mutations. At the hybrids’ segregation received with participation of CCG sources, are formed genotypic classes with absence of semilethal mutations and presence of the active CCG components providing heterosis kept in a number of generations (CCG-effect). In such genotypes, at absence of the harmful mutation, the compensating effect of the co adapted block of CCG genes is realized in positive additive influence on whole organism, raising its general viability. Homozygous CCG-lines differ the improved economic-valuable traits and can be used as a highly productive initial material at plant breeding.

The most reliable way of revealing of the compensated semilethals in a genotype is hybrid analysis of the potential CCG source. At presence of compensated semilethals in F₂ the homozygous semilethal plants with recessive allele will appear.

At cytogenetics researches of one of CCG sources – soybean variety Fora the increase in mitosis infringements in anaphase, reaching 16-20 % is revealed in comparison with 0-7 % at common varieties. The basic type of infringements in mitotic cycle was backlog of one, less often than several chromosomes in process of anaphase movements of chromosomes to poles of a cell.

During researches of CCG-effect at a soybean the wide breeding material which has served by a basis for creation of highly productive varieties has been created. The long-term data of competitive testing have shown convincing advantage on efficiency of hybrid combinations with participation of CCG sources above common varieties. Now three CCG-varieties of soybean are being successfully cultivated occupying more than 60 % of all area under a soybean in the south of Russia.

Key Words: breeding, CCG, semilethal, soybean, transgression,

Introduction

The major and one of most challenges of genetic improvement of a soybean, as well as other agricultural crops, is the further increase of new created varieties’ productivity. It gets a special urgency in modern conditions on a background of the achieved breeding progress and more and more reduced gain of productivity.

In our opinion the perspective way of the problem decision how to increase the soybean productivity is connected to the theory of compensating complexes of the genes, the put forward by V.A. Strunnikov in 1974 (Kaydanov, 1996; Strunnikov, 1997).

According to Strunnikov's concept in a genotype – the carrier of recessive semilethal mutations in the natural or artificial way can collect powerful compensating complex of dominant and codominant genes (CCG), compensating negative influence on organism semi lethal mutations. As a result of hybridization of such genotype with genetically unrelated varieties in F₁
recessive mutant allele passes in a heterozygotic condition and it is not shown, and unary, but the coordinated doze of CCG it is additive influences all hybrid organism, providing powerful heterosis (Kaydanov, 1996; Strunnikov, 1997).

In spite of the fact that Strunnikov's concept has been formulated on the basis of experiments with a silkworm, it can quite make a practical basis for development of methods of the directed selection of parental pairs in breeding of vegetative organisms, including a soybean. The basic problem in practical application of CCG-concept in soybean breeding is presence of the genotypes having full enough compensated semilethal or subvital genes. The soybean represents annual plants, in natural conditions allowing receiving one, seldom two generations in one year (Leschenko et al, 1987). In this connection artificial saturation of semilethal genotype with a complex of compensative genes - equalizers is represented technically bulky and long.

The most effective, in our opinion, is search and allocation of genotypes already with naturally formed CCG during crop microevolution. Certainly, the most reliable way of revealing compensated semilethal is hybrid analysis of hybrid combinations with attraction of potential CCG sources as parents. In this case, an attribute of semi lethal genes the occurrence in homozygous semi lethal genotypes in F2 with not compensated mutant genes will be fixed (Zelentsov, Moshnenko, 2003).

**Materials and Methods**

The studying the phenomenon of compensating CCG effect and to its application in genetic improvement with has been started in VNIIMK in 1993. By results of hybrid analysis allocated the soybean genotypes, possessing semilethal mutations, which are compensated by CCG. Cytogenetics researches of mitosis at CCG sources carried out at magnification ×500-1000. For preparation and colourings squash preparations the standard technique adapted for a soybean was used. Determined CCG sources crossed to the highly productive varieties adapted to local environment. In segregating populations transgressive genotypes are being determined on productivity and estimated.

**Results**

Over 700 segregated hybrid combinations of a soybean created during the period within 1993 on 2002 have been analyzed and parental genotypes, at which in F2 the subvital and semilethal plants have been appeared, were allocated. Thus, as prospective CCG sources the two soybean varieties (Fora and T-245) have been allocated which have been taken as initial forms for the subsequent hybrid analysis and are involved in crossings with the best varieties which are not having semilethal mutations.

The hybrid analysis in varieties Fora and T-245 had been confirmed presence of semilethal recessive mutations, and absence at them visible attributes of decrease in viability or oppression has allowed assuming presence of active CCG-complexes, effective neutralizes the semilethal mutation (Zelentsov, Pendinen, 2001).

As a hypothesis of the reasons of semilethal formation we had been assumed development of infringements at a cellular or subcellular level in soybean plant tissues. With this purpose the mitotic divisions in root apical merystem of 8 soybean varieties have been analyzed (tab. 1). It has been established, that genotypes – carriers of latent semilethal mutations the basic type of infringements mitotic divisions had backlog of separate chromosomes during their divergence to poles of a cell in anaphase, and sometimes with formation chromatid bridges (fig. 1). The critical threshold of frequency of abnormal anaphases occurrence, providing arising of homozygous semi lethal in hybrid populations F2, makes approximately 9-11 % from the general anaphase number.
As a whole, the received data have allowed us to draw a conclusion on presence of relationship between semilethal formation in hybrid populations and the raised amount of abnormal anaphases, and as consequence, opportunities of use as well cytogenetic method for primary selection of potential CCG sources.

The varieties Fora and T-245 as confirmed CCG donors have been crossed to unrelated varieties with genotypes of type ++, 00 (where: “+” is wild (normal) allele, and “0” is CCG absence), adapted to local conditions of growth (fig. 2).

In generation F₁ initial recessive homozygous mutation $ll$ has passed to a heterozygote condition $l+$. The phenotype of F₁ plants such as $l+, Ccg/0$ with unary doze of the CCG complex has been investigated in detail and described further has used as morphological model for an active component of co adaptive CCG block estimation at selection of genotypes of type ++, $c cg2 c cg2$ (where $c cg2$ - is linked inherited components of the CCG complex).

In segregated hybrid population $F_2$ the following phenotypic classes allocated: - homozygotes on semilethal allele such as $ll, 00$ with semilethal phenotype;
- transgressive (heterotic) hybrid genotypes of phenotypic class $+, c cg2_-, ccg2$ - relatives on a morphologic type to reference plants $F_1$ which had active components of the CCG complex and was absent CCG forming semilethal gene;
- hybrid genotypes – carriers as semilethal allele, and elements of the CCG-complex of phenotypic class $ll, c cg2-, c cg2$ - and also hybrid forms with absence of the CCG complex with phenotypic class $++, 00$ which norm of reaction did not leave for extreme values of corresponding traits both parental genotypes.

Allocation in $F_1$ morphological traits for the description of CCG model on all hybrid combinations with participation of CCG sources appeared effective at selection of CCG plants in $F_2$. The analysis of frequencies phenotypic normal and semilethal classes in $F_2$ has shown that in all hybrid combinations with participation of CCG sources it is found out close to 15:1 a ratio. All plants $F_2$ were well divided on 3 morphologically differing groups (fig. 3). All semilethal plants such as $ll, 00$, and also hybrid forms of phenotypic classes $ll, c cg2-, ccg2$ - deleted. Plants of phenotypic class $++, c cg2-, ccg2$ with positive transgressions on the basis of the CCG model, usually occupied 53.0-58.7% from the general amount of analyzed plants, were planted for growing following $F_3$ generations.

In hybrid generations $F_3$-$F_4$ repeated procedure of a visual and morphological estimation of plants, selecting transgressive plants of phenotypic class $++, c cg2-, ccg2-$, and deleted hybrid forms of phenotypic classes $ll, 00$; $ll, c cg2-, ccg2$- and $++, 00$. Posterity selected in $F_4$ CCG plants planted on individual plots for its estimation on genetic uniformity.

In $F_5$ phenotypically homogeneous CCG-lines of genotype $++, c cg2 c cg2$ with positive transgressions to the basic economic valuable traits selected.

**Discussion**

During researches of CCG effect at a soybean we had been created the extensive breeding material which has served by a basis for creation of highly productive varieties. During the period within 1993 on 2002 it has been created more than 120 highly productive CCG-lines. The best on efficiency and other economic valuable traits of the CCG-lines have been transferred on the state test and brought in the state registry of breeding achievements (tab. 2).

The long-term data competitive testing has shown convincing advantage on efficiency of hybrid combinations with participation of CCG sources above usual varieties. Now 5 highly productive CCG-varieties of a soybean are successfully cultivated occupying more than 60% of all area under a soybean in the south of Russia.
Thus, all above-stated allows us to make the conclusion that orientation of breeding programs on a soybean on intensive search and use of genotypes – CCG sources represents a new, perspective way of increase of viability and efficiency of varieties of this crop.

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Tables Legends
Table 1 – Frequency abnormal anaphase in mitosis

<table>
<thead>
<tr>
<th>Variety</th>
<th>The general number of cells in a anaphase stage</th>
<th>Number of normally sharing cells</th>
<th>Amount of infringements in mitosis at a anaphase stage, %</th>
<th>Presence of semilethals in F₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>early anaphase</td>
<td>late anaphase</td>
</tr>
<tr>
<td>Hodgson</td>
<td>5431</td>
<td>5216</td>
<td>30.37</td>
<td>0.59</td>
</tr>
<tr>
<td>(standard)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-245</td>
<td>3677</td>
<td>3188</td>
<td>13.28</td>
<td>0.00</td>
</tr>
<tr>
<td>Fora</td>
<td>7970</td>
<td>6625</td>
<td>14.60</td>
<td>2.27</td>
</tr>
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</table>

Table 2 – The comparative characteristic of some CCG varieties of a soybean, Krasnodar, 1998-2003.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Maturity group</th>
<th>Genetic formula</th>
<th>Productivity, t/ha</th>
<th>The period of vegetation, days*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bystritsa 2</td>
<td>0</td>
<td>++, 00</td>
<td>1.99</td>
<td>108</td>
</tr>
<tr>
<td>(group standard 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delta</td>
<td>0</td>
<td>++, ccg2ccg2</td>
<td>2.60</td>
<td>108</td>
</tr>
<tr>
<td>Hodgson</td>
<td>1.4</td>
<td>++, 00</td>
<td>2.44</td>
<td>125</td>
</tr>
<tr>
<td>(group standard 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vilana</td>
<td>1.0</td>
<td>++, ccg2ccg2</td>
<td>2.72</td>
<td>118</td>
</tr>
<tr>
<td>Renta</td>
<td>1.0</td>
<td>++, ccg2ccg2</td>
<td>2.75</td>
<td>118</td>
</tr>
</tbody>
</table>

* - at latitude 45°

Figure Legends

Figure 1 – Sample of mitotic infringements at anaphase stage, ×1000 (by the example of a variety Fora)
Figure 2 – The breeding of high productive soybean varieties with use of CCG effect

- mutant semi lethal allele; + - wild allele; \textit{Ccg1} - initial CCG; \textit{ccg2} - it is linked inherited polymeric CCG component; 0 - CCG absence; – - phenotypic radical describing anyone condition of allele or a polymeric trait.
Figure 3 – Phenotypic classes of segregated combination $F_2$ Fora × Yug-30 to traits «amount of beans per a plant» and «height of a plant»
Are apomictic maize x *Tripsacum* hybrids a new agricultural crop?

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**Abstract**

Producing apomictic maize would allow to largely cut expenses in this crop’s hybrid seed-farming and considerably advance the solution of the problem of its use in developing countries. For this, we chose here a traditional way to transfer the required trait from a maize related species possessing it. Tetraploid *Tripsacum dactyloides* 2n = 4x = 72 is the closest maize related apomictic species. Hybridisation of tetraploid maize (2n = 4x = 40) with *Tripsacum* was carried out to obtain apomictic maize. Amphiploid F₁ hybrids had 2n = 56 (20Zm +36Td) not very high female fertility and were completely male-sterile. Pollination them by maize allowed to obtain a wide range of apomictic lines with a reduced chromosome number of the wild parent (one and the same group in composition out of 9 chromosomes among lines of different origin). At this stage it is the minimal *Tripsacum* chromosome number providing apomictic reproduction in hybrids. Moreover, our results showed that RFLP markers co-segregating with the trait of asexual seed reproduction are found in six *Tripsacum* linkage groups, and the research continues in this trend. Herewith it is possible to conclude that apomixis is under polygenic control, which elements are widely dispersed over the wild species genome, and due to these reasons, its introgression into diploid maize genome is impossible. However, there is a real possibility to implement rapid and successful improvement of apomictic maize x *Tripsacum* hybrids and producing a new type of cultivated plants. Some apomictic lines with 2n = 39 (30Zm + 9Td) and 2n = 49 (40Zm +9Td) are currently close to maize as for cob size and grain raw number in it on account of multi-year selection. Alongside with this, their male sterility is lower than that of maize, female sterility being a considerable obstacle so far in producing commercial varieties. Nevertheless, maize x *Tripsacum* hybrids have undoubtful advantages over maize as a potential food and fodder crop they have higher: 1) grain lysine and methionine content; 2) content of polyunsaturated fatty acids; 3) protein content in green matter; 4) fibre digestion by animals than that of maize. All the above-enumerated facts allow us to state the fact that the potential value of apomictic maize x *Tripsacum* hybrids is higher than that of maize used worldwide. Besides, they have the whole number of advantages in resistance to biotic and abiotic environmental stresses that cause numerous problems for maize breeders and producers. Our research of maize x *Tripsacum* hybrids showed that this object of investigation, as well as maize, is genetically quite mobile, and it is possible to carry out successful selection for the increase of agronomic indices among them. Thus, not so long ago low female fertility was a considerable problem of hybrids. . We managed to achieve an 80% of cob grain content in 39-chromosome maize x *Tripsacum* hybrids. Based on all the above-formulated, one can assert the thing that producing a new type of agricultural plants is possible.

**Key Words:** Apomeiosis, apomixis, diplospory, gamagrass, imprinting, maize, parthenogenesis

**Introduction**

In 1957, D.F. Petrov published a paper, in which he discussed the possibility of transferring apomixis to corn via intergeneric hybridization with *Tripsacum* (Petrov, 1957). Apomictic F₁ hybrids of maize with *Tripsacum* were developed in the D.F. Petrov’s laboratory in 1964. The next step was reduction of *Tripsacum* chromosomes by pollinating hybrids by maize, and
selection B_{III} with cultivated parent genome amount accumulation with increased B_{II} in offspring. Among the latter, apomictic forms were selected with a reduced number of *Tripsacum* chromosomes and, thus, minimize or, in case of inclusion of *Tripsacum* chromosomes sites carrying genes for apomixis in chromosomes of maize, to exclude the wild parent genetic material at all and develop 20-chromosome apomictic maize (Petrov et al., 1984). An apomictic maize with 20 chromosomes developed (Petrov et al., 1985), but as it has turned out later this was a result of erroneous experiments (Yudin and Sokolov, 1989). At the same time it should be stressed that by Petrov’s group stably apomictically reproducing hybrids of maize with 18 added chromosomes of *Tripsacum* were developed and have been maintained about 40 years now. In this communication we will be telling about complexity of genetical control of diplospory and next step in apomictic corn way –39 chromosomes hybrids (30Zm+9 Td) and their agricultural potential.

**Materials and Methods**

Apomictic corn-gamagrass hybrids 2n=38 (20Zm+18Td) have used for backcrossing by corn and B_{III} hybrids were selecting for next backcross. B_{II} hybrids with reduced number of parents chromosomes were achieved with cytogenetical control by methaphase analysis. Usual 1-bromonaphthalene technique with acetoorsein stain was using to obtain methaphase chromosomes. For segregation analysis F_{1} corn-gamagrass hybrids have pollinated by diploid maize and BC_{1} analyzed morphologically and cytogenetically for type of offspring. The RFLP analysis was made by standard protocol. All statistic analyses were standard.

**Results**

Due to the specificity of the composition of the 38 chromosome offspring of H278 and H32 and the pseudogamous type of diplospory in the series of their generations expected could be: 1) conservation of the apomictic mode of reproduction and maintenance of the genome at the 56-chromosome level; 2) appearance of B_{III}-hybrids and accumulation in this way of maize genomes, strengthening sexualization, and segregating B_{II}-hybrids with a reduction in the number of *Tripsacum* chromosomes; 3) appearance of recombinant maize-*Tripsacum* chromosomes thanks to the rarely occurring meiotic diplospory and their heterologous conjugation due to the synteny of the genomes; 4) segregating new forms of plants owing to the high variability of maize genome; 5) morphologically invisible rapid mutual reorganization of the genetic material typical of allopolyploids, (Leitch and Bennett, 1998, Liu and Wendel, 2002). All supposed events were observed by us at some frequency or other in the series of offspring from backcrosses of the F_{1} (Fig 1). Here we are obliged to specially emphasize that the minimum number of chromosomes necessary to maintain the apomictic mode of reproduction is equal to 9 (Sokolov et al. 1996). Our numerous attempts to reduce one and save apomixis in offspring, was failure.

The results of backcrossing 23 independently produced 46-chromosome F_{1} hybrids (10Zm + 36Td), using diploid corn as the pollen parent. Most of the BC_{1} families (15 of 23) consisted of apomictically produced plants, i.e. they were morphologically homogeneous and had 46 chromosomes like the mother hybrid. Another group of 7 BC_{1} families consisted mostly of apomictically produced offspring, but in addition, they contained 1 to 3 BC_{1} B_{III} hybrids. In this case, as well as in those considered before, parthenogenesis was not 100% penetrate though the ratio of the genomes (1 : 2) was in favour of the apomictic parent. Finally, one BC_{1} family was represented by B_{III} hybrids only, i.e. parthenogenesis had not occurred. The results indicate that, to all appearance, apomeiosis is controlled by one gene or a group of tightly linked genes and has high penetrance. Parthenogenesis, on the contrary, is under the control of many genes and its manifestation depends on genotypic and external factors. Impossibility to obtain apomictic corn
with few genes from gamagrass is obvious from complexity of its genetic control. In this case, we make a parameters comparison of some quantitative traits in hybrids with different ratio of parents genomes in comparison with Wilson F₁ and mother line for understanding commercial value of ones (Table 1).

**Discussion**

When discussing the genetic control of apomixis the researchers proceed from the obvious hypothesis by G.L. Stebbins (1950) of its digenic control - one gene for apomeiosis and another for parthenogenesis. Now debates about the inheritance systems became especially sharp as from the point of view of this trait use in genetic engineering one must exactly know the number, size of its DNA-determinants and their functional control (Mazzucato, 1997). Attempts are currently being made to determine whether apomeiosis and parthenogenesis segregate from each other in backcross generations obtained from hybrids produced between apomicts and sexual forms. We do not consider here all that has been done in this regard (Askier and Jerling, 1992, Nogler, 1984, Mogie, 1992, Savidan, 2000). However, because of our current understanding of the complexity of reproduction and kernel development, a two gene hypothesis is an unacceptable simplification (Carman, 1997, Sokolov et al., 2000). On the base of our results we can claim now about independent genetical control of apospory and parthenogenesis. The genes control of gamagrass diplospory cosegregate with six independent unlinked RFLP markers (Blakey et al., 2001). However, there is a real possibility to implement rapid and successful improvement of apomictic *Tripsacum* x maize hybrids and producing a new type of cultivated plants. Some apomictic lines with 2n = 39 (30Zm + 9Td) and 2n = 49 (40Zm+9Td) are currently close to maize as for cob size and grain raw number in it on account of multi-year selection. Alongside with this, their male sterility is lower than that of maize, female sterility being a considerable obstacle so far in producing commercial varieties. Nevertheless, maize *Tripsacum* hybrids have undoubtful advantages over maize as a potential food and fodder crop they have higher: 1) grain lysine and methionine content; 2) content of polyunsaturated fatty acids; 3) protein content in green matter; 4) fibre digestion by animals than that of maize. All the above-enumerated facts allow us to state the fact that the potential value of apomictic maize *Tripsacum* hybrids is higher than that of maize used worldwide. Besides, they have the whole number of advantages in resistance to biotic and abiotic environmental stresses that cause numerous problems for maize breeders and producers. Our research of maize *Tripsacum* hybrids showed that this object of investigation, as well as maize, is genetically quite mobile, and it is possible to carry out successful selection for the increase of agronomic indices among them. Thus, not so long ago low female fertility was a considerable problem of hybrids. We managed to achieve an 80% of cob grain content in 39-chromosome maize *Tripsacum* hybrids. Based on all the above-formulated, one can assert the thing that producing a new type of agricultural plants is possible.

This research was supported by the Russian Foundation of Basic Research, Grant No. 03-04-49658.

**References**


Figure Legends
Scheme 1. The pedigree of 39-chromosome lines, obtained from the hybrid H278

* We corrected the number of chromosomes in several regions of the scheme (marked by "?"), on the basis of karyological analysis of sib lines, because we found some mistakes in the chromosome counts of the original authors due to imperfect microscopic technique used at that time.
### Tables Legends

Table 1. Quantitative traits in maize-*Tripsacum* hybrids depending on maize (Zm) to Tripsacum (Td) genome ratio

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plant height</th>
<th>Tassel length</th>
<th>Tassel branchlet number</th>
<th>Cob internode length</th>
<th>Cob width</th>
<th>Leaves over top cobs</th>
<th>Cob length</th>
<th>Cob number</th>
<th>Cob row number</th>
<th>Tiller number</th>
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<td>Wilson 2n=20</td>
<td>146.5 ± 2.1</td>
<td>38.9 ± 0.3</td>
<td>8.7 ± 0.2</td>
<td>10.8 ± 0.2</td>
<td>9.5 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>22.6 ± 0.2</td>
<td>14.0 ± 0.2</td>
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<tr>
<td>Tetraploid corn</td>
<td>96.9 ± 1.8</td>
<td>36.1 ± 0.6</td>
<td>9.5 ± 0.4</td>
<td>13.1 ± 0.2</td>
<td>6.9 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>13.2 ± 0.2</td>
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<td>2n=40</td>
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<tr>
<td>Hybrid 2n = 56</td>
<td>157.0 ± 1.5</td>
<td>28.5 ± 0.2</td>
<td>8.4 ± 0.2</td>
<td>14.4 ± 0.3</td>
<td>4.3 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>8.3 ± 0.1</td>
<td>4.0 ± 0.0</td>
<td>7.6 ± 0.5</td>
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<td>(20Zm + 36Td)</td>
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<td>Hybrid 2n = 48</td>
<td>142.7 ± 1.8</td>
<td>33.1 ± 0.4</td>
<td>12.9 ± 0.3</td>
<td>11.1 ± 0.2</td>
<td>6.7 ± 0.1</td>
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<td>11.7 ± 0.2</td>
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<td>4.6 ± 0.3</td>
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<td>(30Zm + 18Td)</td>
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<td>Hybrid 2n = 58</td>
<td>101.2 ± 2.4</td>
<td>27.9 ± 1.2</td>
<td>12.5 ± 0.7</td>
<td>12.6 ± 0.3</td>
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<td>2.6 ± 0.1</td>
<td>10.1 ± 0.3</td>
<td>9.7 ± 0.2</td>
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<td>(40Zm + 18Td)</td>
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<td>Hybrid 2n = 39</td>
<td>174.0 ± 2.3</td>
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<td>2.1 ± 0.1</td>
<td>9.4 ± 0.2</td>
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<td>(30Zm + 9Td)</td>
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<td>Hybrid 2n = 49</td>
<td>141.5 ± 3.3</td>
<td>39.7 ± 0.3</td>
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<td>7.7 ± 0.2</td>
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<td>(40Zm + 9Td)</td>
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<td>Hybrid 2n = 59</td>
<td>150.0 ± 4.8</td>
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<td>12.7 ± 0.5</td>
<td>7.5 ± 0.2</td>
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<td>12.2 ± 0.2</td>
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<td>3.6 ± 0.2</td>
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<td>(50Zm + 9Td)</td>
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