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Male Gametophytic and Sporophytic Screening of Olive Cultivars for Salt Stress Tolerance

A. Soleimani[1], A. R. Talaie[2], M. R. Naghavi[3], and Z. Zamani[2*]

ABSTRACT

Pollen grains as well as whole plants of four olive (*Olea europaea* L.) cultivars were screened for NaCl salinity stress. Different vegetative and physiological indexes of the cultivars were analyzed. Leaf and root Na/K ratio as well as stomatal resistance of plants exposed to salinity proved to be appropriate indexes of whole plant response to salt stress. Whereas, *in vitro* pollen germination percentage served as a reliable index of pollen tolerance to salinity, pollen tube growth did not. A close correlation between pollen (gametophyte) and whole plant (sporophyte) responses to salinity was observed. These parallel responses imply that selection exerted at the gametophytic level could overcome breeding problems encountered at sporophytic level when dealing with adult olive plants.

Keywords: Gametophyte, Olive, Pollen grain, Salinity, Sporophyte, Stomata resistance.

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INTRODUCTION

Olive (*Olea europaea* L.) is an evergreen woody crop plant with great potential for growth in areas where salinity is a major abiotic stress factor for agricultural productivity (Chartzoulakis, 2005). Olive salinity tolerance is a cultivar dependent characteristic (Tattini *et al.,* 1997). In this context serious attempts have been made to identify and improve genotypes (Chartzoulakis, 2005; Marin *et al*., 1995). However, most plant breeding programs are costly and time consuming, especially in olive as a woody and perennial plant (Rugini and Pesce, 2006). The use of new approaches for applying selection pressure at the initial stages of the plant life cycle would facilitate the use of a large population size at the beginning of the breeding program (Hormaza and Herrero, 1996). Breeding efforts have usually been targeted at selecting olive genotypes at whole plant (sporophytic) level by analyzing the behavior of plants in the face of stress (Chartzoulakis, 2005; Tattini *et al*., 1997). Similarity in behavior between the gametophytic and saprophytic phases in relation to different abiotic stresses such as salinity, drought and simulated microgravity (Vasiliy, 2000; Ravikumar *et al*., 2003; DeMicco *et al*., 2006) as well as biotic stress (Dominguez *et al*., 2006) has been assessed. Furthermore, it is possible to analyze the behavior of pollen produced by a single plant against different stress conditions without any need to destroy the plant. *In vitro* pollen selection is one of the most interesting approaches to study the effect of an external stress at gametophytic phase, since the selective pressure is applied

[1] Department of Horticultural Sciences, Faculty of Agriculture, University of Zanjan, Zanjan, Islamic Republic of Iran.
[2] Department of Horticultural Sciences, Faculty of Agriculture, University of Tehran, Karaj, Islamic Republic of Iran.
[3] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Tehran, Karaj, Islamic Republic of Iran.
*Corresponding author, email: zzamani@ut.ac.ir

directly and uniquely to the large male gametophytic population in the same manner as it is applied to plants during screening for salinity tolerance (Tyagi and Rangaswamy, 1993; Martinez-Palle *et al*., 1995). In the present study salinity stress was applied to olive plantlets of different cultivars and to pollen grains obtained from same cultivars as well, to find out whether more tolerant parents would end up with better pollen germination and growth under salt stress conditions.

MATERIALS AND METHODS

Plant Materials and Salinity Treatments

In April 2005, semi-hard wood stem cuttings were taken from 8-year-old olive trees of cvs. Frantoio and Leccino (Italian cultivars) as well as from Rowghani and Zard (Iranian cultivars). Rooted plants were transferred into pots containing a mixture of sand, soil, and leaf mold (1:1:1). Prior to the initiation of the experiment, plants were irrigated weekly and sprayed biweekly by liquid foliar fertilizer (Phosamco 4, Kavin Co. Iran) of a concentration of 2000 ppm. After a lapse of three months the plants were arranged through spilt-plot, based on a completely randomized design while three salinity treatments being applied as: SS0, SS1 and SS2 in which plants were irrigated with salt water containing 0.60 and 120 mM of NaCl, respectively treated with NaCl of 30 mM increments. The different salinity treatments were continued for 35 days.

Stomatal Resistance

At the end of the experiment, stomatal resistance (s cm^{-1}) was assessed using a porometer (AP4, Delta-T Devices; Cambridge-England). Measurements were carried out in the morning (09:00-10:30 a.m.) on 5 to 6th fully expanded leaves from the shoot tip.

Growth Parameters

Relative shoot growth was assessed by evaluating the increase in stem length of plants per each treatment as measured at the beginning and at the end of the salinity treatment. At the end of experiment, total leaf area were determined trough a summation of the individual leaf areas of each plant by use of a leaf area meter (LAM, Delta-T device Cambridge-England). Dry weight of roots, shoots and their ratios were determined destructively at the end of each experiment.

Tissue Mineral Contents

Leaves and roots in each sample were dried up in an oven at 70°C for 48 hours and then ground to a fine powder. Samples were further burned in an electronic oven at nearly 500°C to produce ashes. Na^+ and K^+ contents were determined in ash samples through a 310C digital flame photometer (TAP Co. Tehran-Iran). Ion concentrations were expressed on a tissue dry weight basis.

Salinity Treatments of Olive Pollen Grains

Pollen samples were obtained from orchard grown in olive tree *cvs* Frantoio, Leccino, Rowghani and Zard. Experiments were conducted in tissue culture plates each containing 0.8% (w/v) agar, 15% (w/v) sucrose, and 100 ppm (mg l^{-1}) boric acid through two factorials based on a completely randomized design. Salinity stress was induced by the addition of appropriate quantities of a 2.5M NaCl stock solution to obtain different salt treatment levels corresponding to 0 (Control or T0), 6.25 (T1), 12.5 (T2), 18.75 (T3) and 25 mM (T4). Pre-hydration of stored pollen was performed through incubation in a humid chamber at room temperature for 30 minutes. Pollens were sown using a brush to have them distributed as uniformly as possible onto the plates containing 6 ml of germination medium. Petri dishes were stored at room temperature and in the dark. Sixteen hours later, plates were sprayed with fixative solution [90% (v/v) ethanol 70%; 5% formalin (v/v) and 5% acetic acid (v/v)] and then examined under a microscope. Pollen was considered as germinated when the length of the pollen tube was greater than at least double the pollen grain's diameter (Ravikumar *et al.*, 2003). Germination was quantified as the percentage of germinated pollen per 10 evaluated areas on the culture medium. Pollen tube length was measured employing graded optic.

Data Analysis

Data were analyzed through MSTATC software and means compared through Duncan's multiple range test.

RESULTS

Plant growth was evaluated on the basis of shoot length, total leaf area, dry weight of shoots and root tissues. Shoot growth following initiation of salinity treatments showed significant variations among cultivars and was reduced by 30% and 49% in 60 mM and 120 mM NaCl treatments respectively (Figure 1 A). The lowest and highest reductions were recorded for "Frantoio" and "Leccino" respectively. Salinity caused a decrease in total leaf area of the newly developed leaves in all cultivars with the exception of "Frantoio" in 60 mM treatment (Figure 1 B). Shoot dry weight was reduced by 17% and 36% in 60 and 120 mM NaCl respectively. Root dry weight was significantly decreased (27%) in the 120 mM treatment. The ratio of shoot to root dry weight was significantly higher in control plants than in salt-treated plants (Figure 1 C). Plants of all cultivars exposed to salinity exhibited a significant increase in stomatal resistance (SR) in comparison with

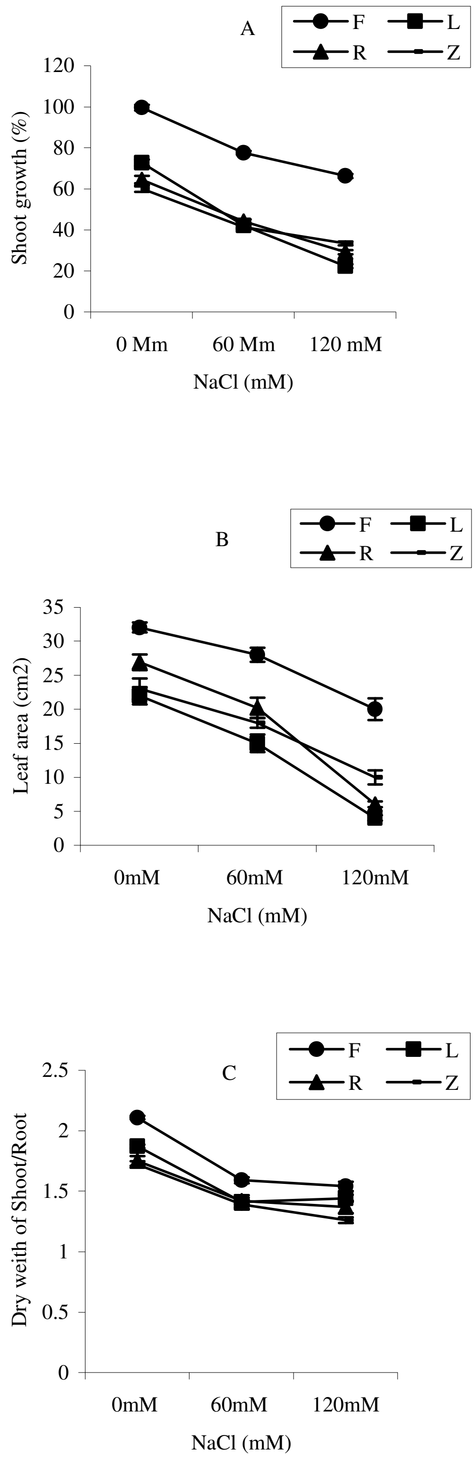

Figure 1. Interaction effects of NaCl salinity stress and olive cultivars on (**A**): Shoot growth length; (**B**): Total leaf area and, (**C**): Shoot/Root dry weight ratio.

control plants with the exception of *cv.* "Frantoio" treated with 60 of mM NaCl. Cultivar differences were more pronounced at high salinity than at either control or medium salinity, with the highest SR being observed in "Leccino" at 120 mM (Figure 2 A). Sodium content in the root tissues increased by 36% and 49% in plants treated with 60 and 120 mM of NaCl respectively.

This increase in leaf tissue was 77% and 90% at 60 and 120 mM salinity respectively (Figures 2 B and C). Genotypic differences were detected in Na^+ accumulation in the root and its subsequent transport into the leaves as on a dry weight basis. "Leccino" showed the highest and lowest Na^+ concentration respectively in leaf and root tissues at 120 mM level of NaCl salinity. While the opposite was detected for

Table 1. Mean comparison of potassium content and Na/K ratio of olive plant tissue under different NaCl salinity levels.

	Physiological traits		
NaCl (mM)	Root K (%D. W.)	Leaf K (%D.W.)	Root Na/K
0	0.87 a	1.16 a	0.74 c
60	0.54 b	1.1 a	1.8 b
120	0.36 c	0.66 b	3.4 a
C.V.	1.85	8.55	14.8

"Frantoio". Salinity in the root zone led to a significant decrease in K^+ content in plant roots at either 60 or 120 mM treatments in all cultivars and in leaf tissues when only at high salinity level (Table 1). Besides "Frantoio", and "Zard" also showed high K^+ content in leaves in comparison with

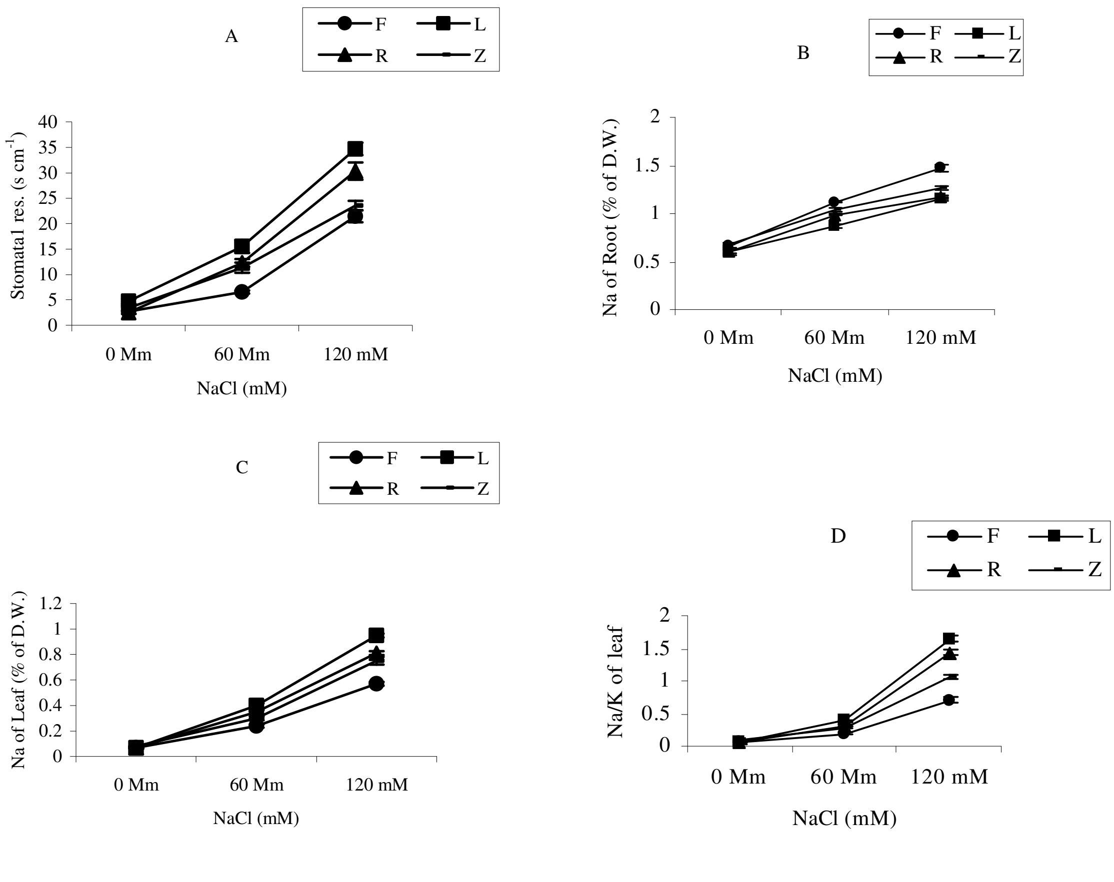

Figure 2. Interaction effects of NaCl salinity stress and olive cultivars on (A): Stomatal resistance; (B): Sodium content of root; (C): Sodium content of leaf and, (D): Na/K ratio of leaf.

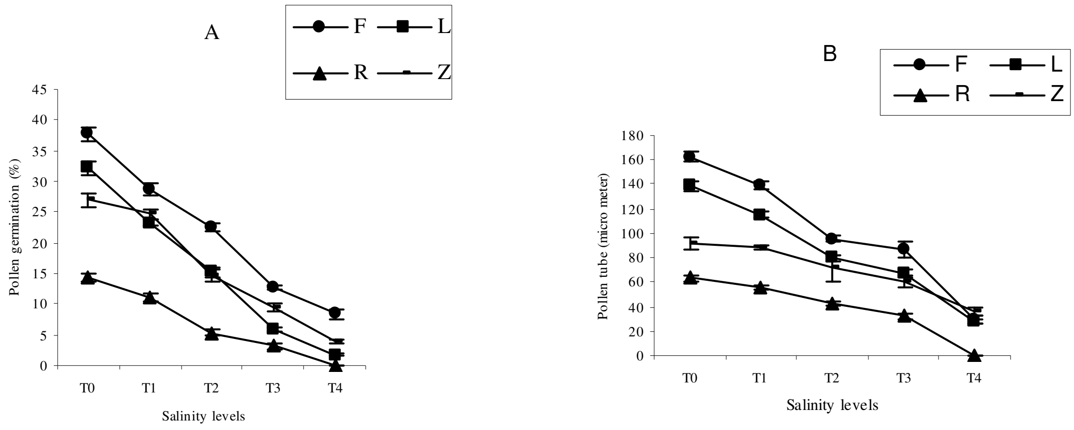

Figure 3. Interaction effects of NaCl salinity stress and olive cultivars on (**A**): Pollen germination and (**B**): Pollen tube length.

"Leccino" and "Rowghani". Significant differences in potassium selectivity transport were observed among cultivars in the Na/K ratio of plant leaves (Figure 2 D). This ratio was high at both 60 and 120 mM treatments in "Leccino" while low in "Frantoio". The influence of NaCl salinity on the *in vitro* germination and on the tube growth of olive pollen were observed among cultivars (Figure 3 A and B and Figure 4). Pollen germination rate decreased by 77%, 94% and 83% at salinity level of T4 in "Frantoio", "Leccino" and "Zard" respectively and while it was completely suppressed and inhibited in "Rowghani". However, pollen tube growth didn't show such a tendency of reduction. Having the lowest reduction rate of pollen germination, "Frantoio" also showed a much higher pollen tube growth suppression under high salinity levels. Pollen survival curves (%germination versus salinity levels) fitted by probit analysis and Lethal Salinity (LS50)(salinity level in which 50% of pollen grains didn't account for as germinated) of each cultivar were plotted. LS50s for "Frantoio", "Zard", "Leccino" and "Rowghani" were 13, 9.5, 6.8 and 5.6 mM of NaCl concentrations in pollen culture media respectively.

DISCUSSION

Although olive is considered as moderately tolerant to salinity stress, however, salinity resistance is a cultivar dependent trait. The results showed a broad genotypic variability to salt stress as indicated by the wide changes in relative shoot growth, tissue dry weight and total leaf area. "Leccino" and "Rowghani" in higher salinity levels showed more reduction of general growth indexes, indicating their sensitivity to salinity as also previously reported (Tattini et al., 1997; Soleimani et al., 2006). Leaf abscission in "Leccino" at 120 mM NaCl contributed to its higher leaf area reduction. Root growth was less affected than shoot in the 60 mM NaCl treatment. A large portion of biomass partitioned to the roots, more evident in "Frantoio", could be considered as a mechanism of the plant to cope with salinity and its osmotic effect in the root zone (Chartzoulakis, 2005). The main limitation of plant growth in salt stressed olive is the low chloroplastic CO_2 concentration, caused by both stomatal and mesophyll conductance reduction (Chartzoulakis *et al.*, 2002). This may explain why "Frantoio" and "Zard", with low stomatal resistance, are apparently more tolerant to salinity. According to Tattini *et al.* (1997), stomatal resistance is more sensitive to salinity in salt resistant

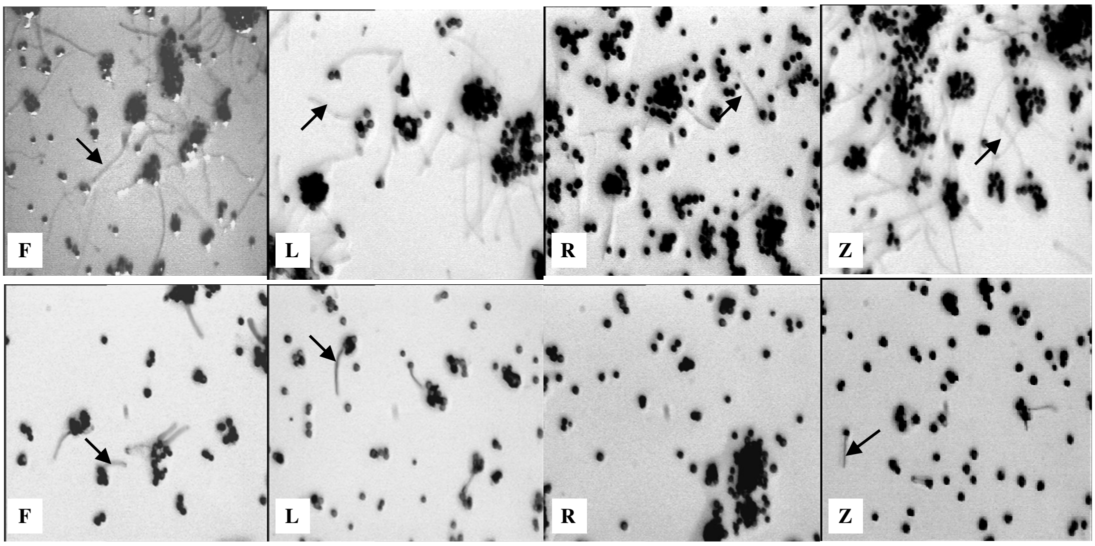

Figure 4. Schematics of pollen germination and pollen tube growth of different olive cultivars (F: Frantoio., L: Leccino., R: Rowghani and Z: Zard) on control (Top) and salt treated (T4= 25 mM NaCl) culture medium (Bottom). Arrows show the pollen tube of the corresponding cultivar.

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cultivar "Frantoio" than in the salt sensitive one "Leccino". The type and age of leaves on which stomatal resistance was assed might be an explanation to this controversy. Measurements in the present work were carried out on fully expanded leaves located at the 5th and 6th nodes from the top. In "Leccino" shoot growth as well as new leaf expansion was suppressed under salinity stress but new and younger leaves at the above mentioned stem nodes were produced in "Frantoio". Moreover, the less pronounced responses of "Frantoio" might have been caused by either a stronger growth in this cultivar during initial stages or to a lower Na^+ accumulation in the leaf, as has been shown as the sensitivity of stomatal apparatus to leaf Na^+ concentration (Gucci *et al*., 1997). Na/K showed the existence of genotypic differences among the cultivars in their ability to exclude the Na^+ ion from the leaves. Low ratio of Na/K in "Frantoio" and "Zard" leaves and high Na^+ concentration in root tissue, indicate the existence of a relatively strong inhibition mechanism of Na^+ transport to aerial part in these cultivars. This is in consistence with current models of ion exclusion in salt-tolerant non-halophytic species (Marcum and Murdoch, 1994). Salt tolerance in citrus is linked with an ability to limit the uptake and/or transport of ions from the root to the shoot (Adnan, 2004). Root potassium content at 60 and 120 mM NaCl and leaf potassium content at 120 mM decreased among cultivars. High tissue Na^+ content along with low K^+ level, showing the well known antagonism between these two elements, has previously been indicated for young olive plants in greenhouse conditions (Bartolini *et al*., 1991). Genotypic screening for the salt tolerance trait among olive cultivars could be achieved based on growth analysis parameters and on two important physiological indexes; Na/K ratio in leaves and stomatal resistance of fully expanded leaves as follows: "Frantoio"> "Zard"> "Rowghani"≥ "Leccino". The present study showed the existence of genetic variability for in vitro pollen germination and tube growth traits and a close correlation between pollen (gametophyte) and whole plant (sporophyte) responses to salinity. According to probit analysis, LS50 of "Frantoio" pollen germination, a cultivar with high tolerance to salinity stress at sporophytic phase, was highest among cultivars (13 mM NaCl). The association between sporophytic and gametophytic responses to salinity has been previously reported (Tyagi and Rangaswamy, 1993; Martinez-Palle *et al*., 1995). Pollen selection using Polyethylene Glycol (PEG) as an agent for osmotic stress induction in sorghum has been shown to influence the growth of resulting plants and the results have demonstrated the transmission of the selected trait from pollen generation to progeny (Ravikumar *et al*., 2003). Pollen germination percentage served as a more reliable index of pollen tolerance to NaCl than pollen tube growth. This is in agreement with the results of Tyagi and Rangaswamy (1993) who screened 11 cultivars from different oilseed brassica species (*B. juncea*, *B. campestris*, *B. carinata*) by using pollen germination percentage instead of pollen tube growth response to salinity. In contrast to this result, in vitro pollen tube growth of inbred maize lines differed significantly for the response to ABA rather than pollen germination (Frascaroli and Tuberrosa, 1993). Based on external stress applied on pollen grains, different traits such as pollen germination, pollen tube growth, changes in reserves or cytochemical organization of grains during pollen tube development might respond accordingly and considered as screening traits (DeMicco *et al*., 2006). While physiological studies revealed higher sensitivity of 'Leccino' to salinity stress than "Rowghani", results of *in vitro* salinity stress on pollen traits showed equal or even less sensitivity of that cultivar than "Rowghani". A part of this controversy arises from the fact that salt tolerance is a highly complex trait and studies to develop selection criteria for salt tolerance at the whole plant level have not only not been promising but even sometimes controversial (Chartzoulakis, 2005). This could be more pronounced in the current study in which younger olive plants (about 4 months-old) were used, as the response of either younger or older plants to external stresses might have been different (Tattini *et al*., 1997). It seems and concluded as well from the current study that pollen germination test under external salt stress could be used as a reliable and low cost technique for at least primary screening of a large population of olive cultivars and genotypes. Later on, the primary selected ones and hence a few numbers could be exposed to future screening pressure by providing cutting and conducting an empirical experiment which is relatively much more time and place consuming as well as more expensive. Moreover, this experiment may provide a clue as how to apply an external abiotic stress on olive pollen maternal plants. As a conclusion, a combination of gametophytic and conventional sporophytic selection criteria should be considered as an effective tool in population improvement programs to achieve higher levels of resistance in a relatively short experimental time.

REFERENCES

1. Adnan, A. Y. 2004. Influence of Salinity on Citrus: A Review Paper. *J. of Cent. Eur. Agric.,* **5**: 263-272.
2. Bartolini, G., Mazuelos, C. and Troncoso, A. 1991. Influence of Na_2SO_4 and NaCl Salts on Survival, Growth and Mineral Composition of Young Olive Plants in Inert Sand Culture. *Adv. Hort. Sci.,* **5**: 73-76.
3. Chartzoulakis, K. S. 2005. Salinity and Olive: Growth, Salt Tolerance, Photosynthesis and Yield. *Agric. Water Manage*., **78**: 108-121.
4. Chartzoulakis, K. S., Loupassaki, M., Bertaki, M. and Anroulakis, I. 2002. Effects of NaCl Salinity on Growth, on Content and CO_2 Assimilation Rate of Ssix Olive Cultivars. *Sci. Hortic.,* **96**: 235-247.
5. DeMicco, V., Scala, M. and Aronne, G. 2006. Effects of Simulated Microgravity on Male Gametophyte of *Prunus*, *Pyrus*, and *Brassica* Species. *Protoplasma,* **228**: 121–126
6. Dominguez, E., Cuartero, J. and Fernandez-Munoz, R. 2006. Sporophytic and Gametophytic Recurrent Selection for Improvement of Partial Resistance to Alternaria Leaf Blight in Sunflower (*Helianthus annuus* L.). *Euphytica,* **147**: 421-431.
7. Finney, D. J. 1971. Probit Analysis, 3rd ed. Cambridge Universit Press, Cambridge. 333pp.
8. Frascaroli, E. and Tuberosa, R. 1993. Effect of Abscisic Acid on Pollen Germination and Tube Growth of Maize Genotypes. *Plant Breed.,* **110**: 250-254

[DOR: 20.1001.1.16807073.2010.12.2.10.8]

9. Gucci, R., Lombardini, L. and Tattini, M. 1997. Analysis of Leaf Water Relations in Leaves of Two Olive (*Olea europaea* L.) Cultivars Differing in Ttolerance to Salinity. *Tree Physiol.,* **17**: 13-21.
10. Hormaza, J. I. and Herrero, M. 1996. Male Gametophytic Selection as a Plant Breeding Tool. *Scientia Horticulture,* **65**: 321-333.
11. Marcum, K. B. and Murdoch, C. L. 1994. Salinity Tolerance Mechanisms of Six C_4 Turfgrasses. *J. Amer. Soc. Hort. Sci.,* **119**: 779-784.
12. Marin, L., Benlloch, M., and Fernandez-Escobar, R. 1995. Screening of Olive Cultivars for Salt Tolerance. *Sci. Hortic.,* **64**: 113-116
13. Martinez-Palle, E., Herrero, M., Aragues, R. and Suelosy Riegos, U. 1995. Salt Response of Seeds and Pollen of Five *Pistachio* Species. *Acta Hort.*, (*ISHS*), **419**: 49-54.
14. Ravikumar, R. L., Patil, B. S. and Salimath, P. M. 2003. Drought Tolerance in Sorghum by Pollen Selection Using Osmotic Stress**.** *Euphytica,* **133**: 371-376.
15. Rugini, E. and Pesce, G. 2006. Genetic Improvement of Olive. *Pomologia Croatica,* **12**: 43-74.
16. Soleimani, A., Zamani, Z., Talaei, A. R. and Naghavi, M. R. 2006. Molecular Characterization of Unknown Potentially Salt Tolerant Olive Genotypes Using RAPD Markers. *J. Sci. Islamic Rep. Iran,* **17**: 107-112.
17. Tattini, M. and Gucci, R. 1999. Ionic Relations and Osmotic Adjustment in Olive Plants under Salinity Stress. *Acta Hort.* (*ISHS*), **474**: 49-54.
18. Tattini, M., Lombardini, L. and Gucci, R. 1997. The Effect of NaCl Stress and Relief on Gas Exchange Properties of Two Olive Cultivars Differing in Tolerance to Salinity. *Plant Soil,* **197**: 87-93.
19. Tyagi, R. K. and Rangaswamy, N. S. 1993. Screening of Pollen Grains *vis-à-vis* Whole Plants of Oilseed Brassicas for Tolerance to Salt. *Theor. Appl. Genet.,* **87**: 343-346.
20. Vasiliy, S. K. 2000. Male and Female Gametophyte Selection of Barley for Salt Tolerance. *Hereditas,* **132**: 1-5.

گزینش اسپوروفیتی و گامتوفیتی مقاومت به شوری در گیاه زیتون

ع. سلیمانی، ع. ر. طلایی، م. ر. نقوی و ذ. زمانی

چکیده

گیاهان کامل چهار رقم زیتون 'فرانتیو'، 'لچینو'، 'روغنی' و 'زرد' تحت تنش شوری ناشی از نمک کلرید سدیم مطالعه و چندین صفات فیزیولوژیکی و رویشی برای گزینش میزان مقاومت آنها یادداشت گردید. صفات فیزیولوژیکی نسبت سدیم به پتاسیم بافت برگ و ریشه و مقاومت روزنه ای از شاخصهای مناسب جهت گزینش ارقام در سطح گیاه کامل مشخص گردید. مطالعه صورت گرفته در سطح دانه گرده در محیط کشت درون شیشه ای حاوی سطوح مختلف شوری نشان داد که درصد جوانه زنی و نه طول لوله دانه گرده می تواند به عنوان معیار گزینشی مناسب برای ارزیابی میزان مقاومت ارقام باشد. رابطه نزدیکی بین پاسخ ارقام به تنش شوری در سطح اسپوروفیت (گیاه کامل) و گامتوفیت (دانه گرده) وجود دارد، بطوریکه درصد جوانه زنی دانه گرده ارقام مقاوم تر نیز بالا بود. وجود چنین همپوشانی بالا در پاسخ سطوح اسپوروفیتی و گامتوفیتی گیاهان، به عنوان گامی موثر و مکمل در پیشبرد و تسریع برنامه های اصلاحی بویژه در درخت زیتون به عنوان یک گونه گیاهی چوبی و چند ساله خواهد بود.