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Effect of Single Node Orientation and Hormone Levels on Lentil *In vitro* Rooting

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Abstract: A culture method was applied as factorial experiment based on completely randomized design using Filip 96-9L genotype of lentil + 0.25 mg L^{-1} Indole butyric acid + different salt levels. Another was done to identify the impact of the different hormone levels on Filip 92-12L genotype including: 0.9 mg L^{-1} Indole acetic acid + 0.2 mg L^{-1} Kinetin, 1 and 2 mg L^{-1} Indole acetic acid + 0.25 and 0.5 mg L^{-1} Indole butyric acid as completely randomized design. In the majority of traits except the length of the plantlet and root, there was significant difference between the two cultures. In all traits, invert culture included better results than normal. It was cleared that for the majority of traits other than the length of plantlet and root fresh weight, there was significant difference among the different hormone levels. 0.9 mg L^{-1} Indole acetic acid + 0.2 mg L^{-1} Kinetin caused the highest rates of the measured traits other than fresh and dry shoot weight. Also, 0.25 mg L^{-1} Indole butyric acid showed the same results except for dry shoot weight.

Key words: Lentil · Indole acetic acid · Indole butyric acid · Kinetin · Single node orientation · *In vitro*

INTRODUCTION

Lentil (*Lens culinaris*) is an important seed legume in the farming systems of the Mediterranean area because lentil seed is a source of high quality protein for human consumption [1, 2, 3]. In addition it is well suited as a rotation crop to replenish soil nitrogen levels. Nevertheless, compared to other legumes such as Pea and Chickpea [4] relatively little biotechnological researches have been performed in lentil and so far, only moderate results have been obtained from this crop [5]. Pulse crops, such as lentils, have long been considered to be recalcitrant to cell and tissue culture and are among the most difficult legumes from which to regenerate whole plants due to problems of root induction [6]. Traditional *in vitro* rooting studies focus on the application of phytohormones especially auxins in order to induce a rooting response. The frequency of root formation in lentil is dependent on cultivar and growing medium [7]. *In vitro* rooting percentage of 40% is considered to be suitable [8]. Although micro-grafting is successful for a number of lentil cultivars [9], a reliable conventional *in vitro* rooting method is highly desirable [10]. Lentil is salt sensitive [11] like many other legume crops and salt stress causes limitation of the under cultivation land and decreases growth and yield of this crop. Selection for salt tolerant varieties would allow cultivating this crop on saline soils or with saline waters which occurring frequently in the Mediterranean area [12] so, prosperity of a lentil breeding program in a large quantity depends on the amount and degree of the available genetic alteration in germplasm. Somaclonal variation is an important factor to initiate genetic alterations in plants that are produced via *in vitro* regeneration systems. Results of the *in vitro* systems reveals that the salt tolerance can be demonstrated in perfect plant so, identifying of salt tolerant plants via tissue culture systems is to be count as a substituting method for classical breeding methods. *In vitro* selection for salt tolerance using shoot apex culture or callus culture can be used as a useful tool for assessment of tolerance in plants [13, 14].

On the other hand, recognition and opportunity to a useful way for micropropagation of the lentil is the first step to perform inter-specific hybridization to extensive narrow genetic base of the plant, among the all transferring resistance genes [15].

The aim of this investigation was to assess the rooting of the shoot nodal segment in lentil as explants in two methods of normal and invert cultures, in salty culture medium and also, examination of different hormone levels to improve *in vitro* rooting of this plant for applying in lentil breeding programs among different selection methods as *in vitro* conditions for salt stress.

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MATERIALS AND METHODS

These experiments included the study of the two methods of direct and invert cultures of nodal segments and examine of the different levels of phytohormones in rooting of nodal segments of the two lentil (*Lens culinaris* Medik) genotypes as follows:

In the first experiment, two methods of culture: direct and invert, were examined. A factorial experiment was carried out based on completely randomized design with four replications using Filip 96-9L genotype and 0.25 mg L^{-1} Indole butyric acid (IBA) in culture medium with different salt levels. Another experiment, as completely randomized design with five replications was conducted to identify the effect of the different hormone levels including: 0.9 mg L^{-1} Indole acetic acid (IAA) + 0.2 mg L^{-1} Kinetin (KN), 1 and 2 mg L^{-1} IAA, 0.25 and 0.5 mg L^{-1} Indole butyric acid (IBA) on Filip 92-12L genotype. Seeds of the Filip 96-9L and Filip 92-12L genotypes were prepared from the Maragheh Research Center for Agriculture and Natural Resources, Iran. The seeds were disinfected with ethanol 70% for about 2 seconds followed by the immersion in sodium hypochlorite 1% for 20 minutes. Sterile distilled water was used to rinse the seeds 3 times. Then the seeds were germinated in sterile Petri-plates in the dark. 4 days after, the germinated seeds were prepared to transfer to the culture tubes. MS[1] medium was used containing 3% sucrose and 0.8% Agar. Medium were adjusted to pH of 5.7 prior to autoclaving at 121°C for 20 minutes. The culture tubes containing 10 mM of MS medium were transferred to the laminar flow under sterile conditions and hygienic points and then germinated seeds were disinfected again and finally, one seed was cultured in each tube. The tubes were barred with cotton and aluminum foil and were tautened around with Para film bands to prevent from pollution. Cultured tubes then were transferred to the growth chamber. The growth chamber conditions were adjusted at 24°C temperatures and 16/8 hours light/darkness photoperiods, respectively (with 300-400 lux light intensity). In the first experiment, six types of MS culture medium with 3% sucrose, supplemented with 0.25 mg L^{-1} Indole butyric acid, containing 6 salt levels: 0, 20, 40, 80, 100 and 120 mM of NaCl were provided and in the second experiment, five types of MS culture medium with 3% sucrose, supplemented with 5 different levels of phytohormones: 0.9 mg L^{-1} IAA+ 0.2 mg L^{-1} KN, 1 and 2 mg L^{-1} IAA, 0.25 and 0.5 mg L^{-1} IBA, were prepared. Four weeks aged plants were picked up in sterile conditions from tubes and were cut to 1 cm segments containing one leaf and bud and each segment was cultured by the inverted orientation in one tube containing 10 mM culture medium. Thereafter, culture tubes were transferred to the culture room with 24°C temperatures and 16/8 hours light/darkness photoperiods, respectively (with 300-400 lux light intensity). 30 days after, some traits were recorded from produced plantlets from single node cuttings in culture medium.

RESULTS AND DISCUSSION

Explant Culture Type: Results of the analysis of variance (data not shown) illustrated that there was significant difference between the two kinds of the cultures ($P < 0.05$) in all of the traits except the length of the plantlet and root and in shoot fresh weight ($P < 0.01$). In addition, there were significant differences between different salt levels in length of plantlet, shoot fresh and dry weight and root dry weight ($P < 0.01$) and root length and root fresh weight traits ($P < 0.05$). The culture type × salt interaction was not significant at all. Mean comparison of culture types (Table 1) showed that in all traits, invert culture included better results and mean comparison of different salt levels showed that all traits were decreased with increasing salt levels (Table 2).

Different Hormone Levels: Analysis of variance of the hormone experiment cleared that for the majority of traits except the length of plantlet and root fresh weight; there was significant difference among the different hormone levels ($P < 0.05$). Results of the mean comparisons showed that 0.25 mg L^{-1} IBA and 0.9 mg L^{-1} IAA+ 0.2 mg L^{-1}KN had the better results (Table 3).

In vitro techniques are important tools for modern plant improvement programs. The ability to regenerate seed-bearing plants is crucial to the successful application of *in vitro* methods with regard to the plant breeding [16]. In a research, nodal segments of lentil with an axillary bud cultured in an inverted orientation (apical end in the medium) showed higher rooting frequencies than explants cultured in a normal orientation and the highest rooting percentage and average number of shoots regenerated per explants, were obtained from explants placed in inverted orientation on MS medium with 3%

[1] Mourashige and Skoog

Table 1: Mean comparisons of the culture type using Tukey test at (P <0.5)

Culture type	Plant length	Root length	Fresh shoot weight	Dry shoot weight	Fresh root weight	Dry root weight
Normal oriantation	3.2	0.4	54.5	4.6	0.4	0.2
Invert orientation	3.5	0.5	73.6	4.7	1.7	0.6

Data with the same letters have no significant difference to each other

Table 2: Mean comparisons of the different salt levels using Tukey test at (P <0.5)

Salinity	Plant length	Root length	Fresh shoot weight	Dry shoot weight	Fresh root weight	Dry root weight
0	4.7^{A}	1.0^{A}	81.9^{A}	6.2^{A}	2.6^{A}	1.0^{A}
20	4.1^{B}	0.7^{AB}	76.3^{B}	5.5^{B}	1.7^{B}	0.6^{AB}
40	3.8^{B}	0.7^{AB}	65.6^{C}	5.5^{B}	1.5^{A}	0.5^{BC}
80	2.6^{C}	0.3^{BC}	55.1^{D}	3.8^{C}	0.4^{C}	0.2^{CD}
100	2.5^{C}	0.0^{C}	51.9^{E}	2.7^{D}	0.0^{D}	0.0^{D}
120	2.3^{C}	0.0^{C}	51.9^{F}	2.3^{E}	0.0^{D}	0.0^{D}

Data with the same letters have no significant difference to each other

Table 3: Mean comparisons of the phytohormones using Tukey test at (P <0.5)

Culture medium	Plant length	Root length	Fresh shoot weight	Dry shoot weight	Fresh root weight	Dry root weight
+0.9 mg l^{-1} IAA 0.2 mg l^{-2} KN	3.7^{A}	1.5^{A}	50.4^{C}	5.7^{B}	2.6^{A}	0.5^{AB}
1 mg l^{-1} IAA	3.3^{BC}	0.7^{B}	70.6^{A}	5.7^{B}	1.8^{A}	0.2^{B}
2 mg l^{-1} IAA	3.1^{C}	0.6^{BC}	68.8^{B}	5.5^{B}	2.0^{A}	0.2^{B}
0.25 mg l^{-1} IBA	3.6^{AB}	1.4^{A}	52.6^{C}	6.6^{A}	2.9^{A}	0.6^{A}
0.5 mg l^{-1} IBA	2.6^{D}	0.0^{C}	42.8^{E}	4.3^{C}	0.0^{B}	0.0^{C}

Data with the same letters have no significant difference to each other

sucrose, supplemented with 5 µM Indole acetic acid (IAA) and 1 µM Kinetine (KN) [16]. The conclusion from the mentioned work is that the improved rooting of the inverted microcuttings was caused by the change in the polarity of shoot. Recent studies on several Australian plants have shown that the medium hypoxia is a critical limiting factor in the *in vitro* rooting process. Newell *et al*. [17] demonstrated that the hypoxic conditions at the proximal end of the microcuttings placed in the Agar rooting medium not only limited root initiation but also root elongation. Medium aeration at the proximal end of the microcutting is more important than shoot orientation for *in vitro* rooting of lentil microcuttings [10]. As we know, Plant tissue culture is a cornerstone of modern lentil breeding programs including the raising of inter-specific hybrids, genetic engineering and haploid induction. It is full of advantage to have a general effective rooting strategy that dose not require individual optimization for each genotype [18]. Rooting of *in vitro* shoots is very important. Microsperma of lentil genotypes requires auxines in order to initiate root on shoots and clears that the lentil genotypes had different *in vitro* rooting responses [19, 7]. The results of *in vitro* salt screening have shown that the length of the potato plantlets is decreased with increasing salt levels and salinity affected shoot growth [20, 21]. Evers *et al*. [22] reported that the most effect of salt stress is on root growth. Farhatullah and Raziuddin [20] and Velasques [23] showed that shoot fresh weight is decreased with increasing salt levels. Morpargo *et al*. [24] reported various responses of different cultivars of lentil to salt stress and that the salt stress affected the growing traits same to our studied traits. It has been found that high levels of salt decreased root fresh weight [25, 23] that is in accordance with the findings of the present work.

CONCLUSION

We found that the two kinds of the cultures differed from each other in terms of all traits except the length of the plantlet and root. Also, there were significant differences between the salt levels for the length of plantlet and root. Moreover, fresh and dry weights of the shoot and root showed significant differences. For the majority of traits except the length of plantlet and root fresh weight, different hormone levels showed significant

difference. It was found that application of 0.25 mg L^{-1} IBA and a combination of 0.9 mg L^{-1} IAA+ 0.2 mg L^{-1} KN treatments were superior to the other hormone levels for the measured traits, as well.

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