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The Effects of Different Concentrations of Nitrogen Sources on Growth of Micropropagated Potato Cultivars

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Abstract
The use of nitrogen in nutrient media is essential for growth and helps identify highly productive media for potato (*Solanum tuberosum* L.) micropropagation and adventitious shoot formation. Three potato cultivars (Agria, Marfona and Savalan cultivars) were examined for their growth response with medium containing four different levels of KNO_3 (1, 1.25, 1.5 and 1.75 times as much 1.9 g l^{-1}) and NH_4NO_3 (1, 1.25, 1.5 and 1.75 times as much 1.65 g l^{-1}). The morphogenic responses of cultivars were evaluated via measurement of root length, number of roots, shoot length, number of shoots and number of nodes per plantlet. Results indicated that the cultivars differed in their response to nitrogen source types and different concentrations. The results of both KNO_3 and NH_4NO_3 experiments indicated that N_3C_1 and N_3C_2 (2.85 g l^{-1} KNO_3 and 2.475 g l^{-1} NH_4NO_3 in Agria and Marfona, respectively) had the longest shoot length. Cultivars Agria and Marfona at 2.375, 2.85 and 3.325 g l^{-1} KNO_3 produced the shortest root length, but cultivar Savalan in all KNO_3 levels had the highest root length. Our findings indicated that the third concentration of both nitrogen sources (KNO_3 and NH_4NO_3) were better than other nitrogen concentrations in all cultivars for number of shoots and number of nodes. Therefore, nitrogen source media should have significant utility for shoot or other important traits in potato *in vitro* culture conditions.

Keywords: *In vitro* nitrogen source; Micropropagation; *Solanum tuberosum* L.

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important crops in the world. Genetic analysis of cultivated and wild species of potato has indicated that southern Peru is the origin of potatoes (Spooner *et al*. 2005). Potato in Iran is cultivated on 180,000 hectares, with 5400000 tons of production (FAO 2013). The yield of potato in Iran is relatively low (about 267,000 kg ha^{-1}) compared with the countries with high mean yields (44,757 and 41811 kg ha^{-1}, p roduced in Germany and US, respectively (FAO 2013). High mean yield and good quality are the most important objectives in Iran's potato breeding programs.

Potatoes are propagated in vegetative form and cultivated as clones (Hoque *et al*. 2010). Although investigations into improving macro-propagation procedures continue, they have lost some impetus in recent years, with the continued extension of tissue culture methods (George and Debergh 2008). The potato is one of the important model plants for tissue culture purposes (Asghari-Zakaria *et al*. 2007). Due to its high regeneration potential, seed tuber production is done extensively through tissue culture. The *in vitro* production of potato is mainly done to maintain genetic stability and mass tuberization for virus-free potato production (Wang and Hu 1982). The production of healthy potato clones combined with *in vitro* procedures have become an

important part of potato seed production, resulting in higher quality seed tubers (Jones 1994). *In vitro* plant tissues are grown on an artificial medium, which supply the necessary macro and micro elements for growth and development (Hashem-Abadi and Kaviani 2010; Motamedi *et al*. 2011).

Nitrogen is an important nutrient for optimizing potato yield and quality. Different media for potato tissue culture have different nitrogen types (nitrogen and nitrate) and concentrations. Morphogenesis is affected by the amount of nitrogen in the medium. The importance of the relative proportions of NO_3^- and NH_4^+ has been demonstrated during indirect morphogenesis and the growth of regenerated plants (George and de Klerk 2008). Atypical growth due to improper balance of nitrate and ammonium has been reported in plants (Pais and Casal 1987; Moriguchi and Yamaki 1989; Grimes and Hodges 1990). The requirements for both forms of nitrogen can only be determined by a carefully controlled experiment. Comparison of the growth response to different media allows some conclusions about the influence of quantitative or qualitative differences in N content.

The ratio of NO_3^-: NH_4^+ required for various purposes is somewhat known. Root growth is promoted by NO_3^- and depressed by NH_4^+, while media containing only nitrate nitrogen are used for culturing the shoots of some plants (George and de Klerk 2008). Although roots are able to absorb nitrate ions from solutions, they may not be able to do the same under the *in vitro* condition for other parts (cells, tissues and organs). It has been reported that shoot explants that are grown without NH_4^+ can become chlorotic, while adding a bit of NH_4^+ to the medium at the final proliferation (start of rooting stage) may result in more developed plants with green leaves (Zimmerman 1981; Piagnani and Eccher 1988).

The effect of different nitrogen sources on growth and morphogenesis of some potato genotypes was investigated by Del Avila *et al.* (1994). The authors concluded that the quality of the nitrogen source affects both the growth and morphogenesis of micro-propagated potato genotypes. Although the effects of different sources of nitrogen on *in vitro* growth characters of some crops [Tsai and Saunders (1999) in sugerbeet (*Beta vulgaris* L.); Sood *et al*. (2002) in radish *(Raphanus sativus* L.); Sotiropoulos *et al.* (2005) in apple (*Malus domestica* Borkh.); Hajnajari *et al.* (2008) in wild cherry (*Prunus avium* L.); Villamor (2010) in ginger (*Zingiber officinale* Rosc.)] have been investigated, but this phenomenon has not been studied in potato tissue culture except by Del Avila *et al.* (1994). In the current work, the effects of NO_3^- and NH_4^+ as sources of nitrogen in medium were investigated on three important potato cultivars.

Materials and Methods

Three commercially important potato cultivars (Agria, Marfona and Savalan) were used in this study. Agria was originated from Germany and has yellow and deep yellow fresh primary tuber color, a high resistance to the most potato viruses and a very high yield potential at the early harvest.

Marfona was originated from the Netherlands and has a light yellow fresh primary tuber color, a medium resistance to most potato viruses and a very high yield potential at the early harvest. Savalan was originated from Iran and has light yellow and yellow primary fresh tuber color, a medium resistance to most potato viruses and a high yield potential at the early harvest. Virus-free potato plants were produced from the virus-free minitubers of these cultivars. They were cultured under controlled conditions in pots containing soil, peat moss and fine sand at equal proportions (1:1:1). These plants were micropropagated by a single node culture in a free-hormone MS, and the new single node explants from the regenerated plant were used to investigate the effect of source and concentration of nitrogen.

The plant material for *in vitro* culture was surface sterilized by spraying with 70 % (v/v) ethanol for 30 seconds followed by a solution of 1% sodium hypochlorite for 5 minutes and washing three times with distilled water. One explant was cultured separately in a 20 × 200 mm tube containing 20 ml free- hormone MS medium. To investigate the effect of different nitrogen sources on plantlet growth, two experiments were performed with four levels of KNO_3 (1, 1.25, 1.5 and 1.75 times as much 1.9 g l^{-1}) and NH_4NO_3 (1, 1.25, 1.5 and 1.75 times as much 1.65 g l^{-1}). The regenerated plantlets were produced at 22 ºC, a 16 hr photoperiod and 6000 lux light intensity for 1 month. Finally, these plantlets were used in the experiment. Five traits, including root length, number of roots, shoot length, number of shoots and the number of nodes per plantlet, were measured.

The experiments were performed according to a factorial experiment using a completely randomized design layout with 10 replications. The first factor was different nitrogen sources (KNO_3 and NH_4NO_3), the second factor was different levels of nitrogen concentration and the third factor was the three potato cultivars. Primary statistical analyses such as the normality test (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test) were conducted. For the data that did not fit a normal distribution, a data transformation process (logarithmic) was used. After analysis of variance (ANOVA), the means were compared using the least significant differences procedure. For non-normal datasets, the nonparametric ANOVA using the Kruskal-Wallis test and nonparametric mean comparisons using the Mann-Whitney test were done. The associations among traits were studied using Spearman's rank correlation (Steel and Torrie 1980). All the statistical analyses were carried out using SPSS version 14 (SPSS Institute 2004)

Results and Discussion

The results of the Kolmogorov-Smirnov normality test for the measured traits indicated that only the root length had data normality (Table 1). Different data transformations were applied to the non-normal traits, but only the shoot length was normal via logarithmic transformation in both experiments (Table 1). The results of the Levene's test for homogeneity of variances were similar to the Kolmogorov-Smirnov normality test (data are not shown). Therefore, conventional parametric statistics were used to analyze the root and shoot length while alternative nonparametric statistics

were utilized to analyze the remaining traits including number of roots, number of shoots and number of nodes in separate analyses for KNO_3 and NH_4NO_3.

Table 1. Normality test parameters for measured traits of micropropagated potato cultivars

Statistics		SL	RL	NR	NS	NN	Log-SL
Parameters	Mean	7.390	5.934	5.446	2.900	6.329	0.794
	Std. Deviation	3.262	0.458	0.718	1.160	2.113	0.090
	Absolute	0.171	0.099	0.312	0.381	0.224	0.187
Most Extreme Differences	Positive	0.157	0.058	0.312	0.381	0.224	0.187
	Negative	-0.171	-0.099	-0.213	-0.219	-0.135	-0.076
Kolmogorov-Smirnov Z		2.656^{**}	1.540^{ns}	4.830^{**}	5.904^{**}	3.476^{**}	1.898^{ns}

^{ns} Non-significant; ** Significant at 0.01 probability level; SL= shoot length; RL= root length; NR= number of roots; NS=number of shoots; NN= number of nodes per plantlet

Based on the analysis of variance, the coefficient of variation (CV) was 3.54% for shoot length and 7.07% for root length. These low CV values indicate a good index of reliability. (Table 2). The main effects of nitrogen source type (N), nitrogen level (L), and cultivar (C) were highly significant at 0.01 probability level for shoot length. Also, the N × C and N × C interactions for shoot length were highly significant at 0.01 probability level, while the L × C and N × L × C interactions were not significant (Table 2). Regarding the significance of both N × L and N × C interactions, the mean comparison of main effects was not logical and so the treatment combinations (N × L × C) were compared via LSD procedure. For root length, the main effects of nitrogen level (L), and cultivar (C) were highly significant at 0.01 probability level, but the main effect of nitrogen source type (N) was not significant. Except N × L interaction, all of the remained interactions (N × C, L × C and N × L × C) were not significant for root length (Table 2). The effects of different concentrations and sources of nitrogen on important traits of plant tissue culture have already been reported by Gerenda and Sattelmacher (1999) in tomato and potato and Hajnajari *et al*. (2008) in wild cherry.

Table 2. Analysis of variance for the effect of nitrogen sources and concentrations on shoot and root length of micropropagated potato cultivars

SOV	df	Shoot length	Root length
Nitrogen (N)	1	0.15804^{**}	0.60000^{ns}
Level (L)	3	0.15728^{**}	1.31472^{**}
Cultivar (C)	2	0.04248^{**}	1.95104^{**}
N × L	3	0.27598^{**}	0.64944^{*}
N × C	2	0.09845^{**}	0.00262^{ns}
L × C	6	0.00130^{ns}	0.22510^{ns}
N × L × C	6	0.00152^{ns}	0.06690^{ns}
Error	216	0.00079	0.17587
CV (%)		3.54	7.07

^{ns} Non-significant; *, ** Significant at 0.05 and 0.01 probability levels, respectively

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For the similarity of presentation, the N × L × C treatment combinations were shown for both measured traits (Table 3). It indicated that $N_1L_1C_3$ (10.350), $N_1L_3C_1$ (9.847) and $N_1L_4C_2$ (10.850) had the highest shoot length, while $N_1L_1C_1$ (0.239), $N_1L_2C_2$ (0.337), $N_2L_1C_1$ (0.477), $N_2L_2C_1$ (0.292) and $N_2L_4C_1$ (0.413) combinations had the lowest shoot length (Table 3). In other words, all cultivars (Agria, Marfona and Savalan) produced the longest shoot length in the KNO_3 nitrogen source, but at different concentrations. The LSD test for root length showed that $N_1L_1C_2$, $N_1L_2C_1$, $N_1L_2C_3$, $N_1L_3C_1$, $N_1L_3C_2$, $N_1L_3C_3$, $N_1L_4C_3$, $N_2L_1C_2$, $N_2L_1C_3$, $N_2L_2C_2$, $N_2L_2C_3$, $N_2L_3C_1$, $N_2L_3C_3$, $N_2L_4C_2$ and $N_2L_4C_3$, had the longest root length (Table 3). It seems that different levels of studied factors (nitrogen types, nitrogen concentrations and cultivars) did not affect root length very much and all cultivars showed considerable long roots in some concentration of nitrogen sources for both nitrogen types. The nitrogen supply, as well as other nutrients, usually affect the *in vitro* culture growth and micropropagation of plants, especially the potato, and the effects depend on the cultivar and the hormonal balance (Danci and Danci 2008). Evans (1993) has reported that the nitrogen levels of MS medium were too high for micropropagation regarding leaf area and shoot length in potato. Furthermore, potato culture is a

Table 3. Mean of different treatment combinations for shoot and root length of micropropagated potato cultivars

Nitrogen	Level	Cultivar	Shoot length	Root length
KNO_3	1	Agria	0.239 H	0.038 E
KNO_3	1	Marfona	7.015 DEFG	5.865 ABCD
KNO_3	1	Savalan	10.350 AB	5.760 BCD
KNO_3	1.25	Agria	6.617 DEFG	6.033 ABC
KNO_3	1.25	Marfona	0.337 H	0.054 E
KNO_3	1.25	Savalan	6.513 DEFG	5.953 ABCD
KNO_3	1.5	Agria	9.847 ABC	5.917 ABCD
KNO_3	1.5	Marfona	5.467 FG	5.943 ABCD
KNO_3	1.5	Savalan	5.580 FG	6.033 ABC
KNO_3	1.75	Agria	7.517 DEF	5.777 BCD
KNO_3	1.75	Marfona	10.850 A	5.603 D
KNO_3	1.75	Savalan	7.767 CDEF	6.123 AB
NH_4NO_3	1	Agria	0.477 H	0.077 E
NH_4NO_3	1	Marfona	8.040 CDE	5.799 ABCD
NH_4NO_3	1	Savalan	6.762 DEFG	6.105 ABC
NH_4NO_3	1.25	Agria	0.292 H	0.047 E
NH_4NO_3	1.25	Marfona	7.665 CDEF	5.855 ABCD
NH_4NO_3	1.25	Savalan	5.900 EFG	6.150 A
NH_4NO_3	1.5	Agria	7.742 CDEF	5.850 ABCD
NH_4NO_3	1.5	Marfona	8.415 BCD	5.742 CD
NH_4NO_3	1.5	Savalan	7.625 CDEF	6.060 ABC
NH_4NO_3	1.75	Agria	0.413 H	0.066 E
NH_4NO_3	1.75	Marfona	6.400 DEFG	5.950 ABCD
NH_4NO_3	1.75	Savalan	4.810 G	6.090 ABC

Means followed by the same letter(s) are not significantly different at 0.05 level of probability

Journal of Plant Physiology and Breeding
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genotype-dependent trait and can be changed by different sucrose levels in the micropropagation medium (Foulger and Jones 1986; Kuria *et al*. 2008; Baciu *et al*. 2009).

Based on the nonparametric analysis of variance (Kruskal-Wallis nonparametric test), the chi-square statistics indicated that for traits such as number of shoots and number of nodes per plantlet in the KNO_3 experiment and for the number of roots, number of shoots and number of nodes in the NH_4NO_3 experiment the differences among treatment combinations (N × C) were significant but no significant differences were obtained for number of roots in the KNO_3 experiment (Table 4). The mean ranks of each N × C combination are given in Table 4. The results suggest that the nitrogen level and type are important and could be expected to influence plant morphology and micropropagation efficiency. Also, changing the nitrogen level and type changed a variety of micropropagation characteristics. Similar findings have been reported by other authors in potato (Evans 1993; Del Avila *et al*. 1994; Baciu *et al*. 2009).

The results of Mann-Whitney nonparametric test for comparing all paired treatment combinations are given in Table 5. The treatment combinations N_3C_1, N_3C_2 and N_3C_3 with four shoots were more favorable than the other treatment combinations in the KNO_3 experiment (Table 5). The cultivar type did not affect the number of shoots, and 2.85 g L^1 KNO_3 produced more shoot number than the other concentrations. Similar to the number of shoots, the treatment combinations N_3C_1 (2.85 g l^{-1} and Agria), N_3C_2 (2.85 g l^{-1} and Marfona) and N_3C_3 (2.85 g l^- and Savalan) with 8.4, 8.6 and 8.5 nodes, respectively, were more favorable than the other treatment combinations in the KNO_3 experiment. The

Table 4. Mean rank of nitrogen×cultivar combinations, averaged over micropropagated potato cultivars, and chi-square statistics of Kruskal-Wallis nonparametric test

	KNO_3			NH_4NO_3		
Combination	NR	NS	NN	NR	NS	NN
N_1C_1	46.6	44.0	35.8	59.7	29.0	19.8
N_1C_2	57.8	53.0	36.3	70.9	35.0	18.9
N_1C_3	70.4	44.0	32.8	84.5	29.0	17.0
N_2C_1	60.0	44.0	65.3	26.3	29.0	66.6
N_2C_2	68.2	44.0	68.8	39.4	29.0	69.3
N_2C_3	66.0	48.5	65.8	66.8	32.0	67.9
N_3C_1	65.2	105.5	104.8	57.7	105.5	101.2
N_3C_2	60.0	105.5	106.3	66.8	105.5	107.1
N_3C_3	60.0	105.5	105.5	57.7	105.5	108.2
N_4C_1	63.0	44.0	35.8	62.9	75.5	45.6
N_4C_2	57.0	44.0	36.3	72.0	75.5	53.6
N_4C_3	51.8	44.0	32.8	61.6	75.5	51.1
df	11	11	11	11	11	11

Chi-Square	5.05^{ns}	112.07^{**}	89.36^{**}	26.36^{**}	116.84^{**}	101.79^{**}

^{ns} Non-significant; ** Significant at 0.01 probability level; NR= number of roots; NS=number of shoots; NN= number of nodes per plantlet

minimum number of nodes for N_1C_1, N_1C_2, N_1C_3, N_4C_1, N_4C_2 and N_4C_3 treatment combinations in the KNO_3 experiment is shown in Table 5. In other words, N3 level of KNO_3 produced more nodes, while N1 and N4 levels of nitrogen produced fewer nodes. In general, for all cultivars, N3 level of KNO_3 produced the most favorable values for number of roots, number of shoots and number of nodes.

According to the Mann-Whitney test, treatment combinations of N_1C_2, N_1C_3 and N_4C_2 had the highest value for the number of roots in the NH_4NO_3 experiment, while N_2C_1 and N_2C_2 had the lowest value for this trait (Table 5). The treatment combinations N_3C_1, N_3C_2 and N_3C_3 had higher numbers of shoots, whereas N_1C_1, N_1C_3, N_2C_1 and N_2C_2 had the minimum number of roots in the NH_4NO_3 experiment. For the number of nodes, N_3C_2 and N_3C_3 treatment combinations had the highest values and N_1C_1, N_1C_2 and N_1C_3 had the lowest values (Table 5). In general, Agria and Marfona at N1, N2 and N3, and Savalan cultivar at N1 and N2 showed highest values for the number of roots. All three cultivars showed the highest values for the number of shoots and number of nodes at N3 level (2.85 g l^{-1}) of KNO_3 and NH_4NO_3. Significant differences among cultivars were observed for number of shoots and number of roots in some N concentrations for the NH_4NO_3 experiment. These results demonstrated the effect of the genotype on the *in vitro* "cultivability" capacity and over micropropagation capacity in the *in vitro* tissue culture conditions under exogenous phytohormones influence (Ranalli 1997; Danci and Danci 2008).

Table 5. Results of paired mean comparison between treatment combinations, averaged over micropropagated potato cultivars using Mann-Whitney nonparametric test

Combination	KNO_3			NH_4NO_3		
	NR	NS	NN	NR	NS	NN
N_1C_1	5.3 A	2.0 B	4.6 C	5.3 B	2.0 D	4.6 CD
N_1C_2	5.6 A	2.2 B	4.6 C	5.6 A	2.2 C	4.6 CD
N_1C_3	5.8 A	2.0 B	4.5 C	5.8 A	2.0 D	4.5 CD
N_2C_1	5.6 A	2.0 B	5.5 B	4.5 C	2.0 D	6.5 B
N_2C_2	5.8 A	2.0 B	5.6 B	4.8 C	2.0 D	6.6 B
N_2C_3	5.8 A	2.1 B	5.5 B	5.4 AB	2.1 C	6.6 B
N_3C_1	5.7 A	4.0 A	8.4 A	5.2 B	5.0 A	9.9 AB
N_3C_2	5.6 A	4.0 A	8.6 A	5.4 AB	5.0 A	10.7 A
N_3C_3	5.6 A	4.0 A	8.5 A	5.2 B	5.0 A	10.8 A
N_4C_1	5.7 A	2.0 B	4.6 C	5.3 B	4.0 B	5.7 CB
N_4C_2	5.5 A	2.0 B	4.6 C	5.5 A	4.0 B	6.0 CB
N_4C_3	5.4 A	2.0 B	4.5 C	5.3 B	4.0 B	5.9 CB

Means followed by the same letter(s) are not significantly different at 0.05 level of probability; NR= number of roots; NS=number of shoots; NN= number of nodes per plantlet

KNO_3 was better than NH_4NO_3 for shoot length. The second and third levels of NH_4NO_3 concentration were better than KNO_3 for number of roots per plant. The different concentrations of KNO_3 were better than those of NH_4NO_3 for shoot length in Agria and Marfona. The number of roots were increased by using NH_4NO_3 in Agria. The KNO_3 concentration increased the number of shoots in Marfona and Savalan. The number of nodes for KNO_3 were better than those of NH_4NO_3 in all cultivars, but there was no significant difference between KNO_3 and NH_4NO_3 concentrations for root length in the three studied cultivars. In contrast to our results, nitrogen in the form of NH_4^+ instead of NO_3^- gave greater shoot length and leaf number in the micropropagation of the three potato cultivars (Del Avila *et al*. 1994). Wetherell and Dougall (1976) evaluated the effect of different sources of nitrogen on growth of wild carrot and declared that a reduced nitrogen source is required as a supplement to nitrate, for tissue growth in *in vitro* conditions. In contrast, Evans (1993) has suggested that the observed variations in potato tissue culture were a result of changes in the nitrogen level rather than changes in the ammonium to nitrate ratio.

To explore association among the measured traits, Spearman's rank correlation was calculated for both KNO_3 and NH_4NO_3 experiments (Table 6). There was a highly significant positive correlation between shoot length and the number of shoots in both experiments. Also, highly significant positive correlation was observed between the number of shoots and the number of nodes in both experiments (Table 6). Futhermore, a highly significant positive correlation was observed between shoot length and the number of nodes in the KNO_3 experiment. The correlation coefficient of shoot length with root length and also root length with number of nodes in the NH_4NO_3 experiment was negative and significant (Table 6).

Table 6. Correlation coefficients between various traits of micropropagated potato cultivars

	KNO_3				NH_4NO_3			
	SL	RL	NR	NS	SL	RL	NR	NS
LR	-0.50^{ns}				-0.84**			
NR	0.05^{ns}	-0.08^{ns}			-0.50^{ns}	0.41^{ns}		
NS	0.72**	-0.03^{ns}	0.11^{ns}		0.60*	-0.33^{ns}	0.01^{ns}	
NN	0.93**	-0.32^{ns}	0.20^{ns}	0.71**	0.91**	-0.74**	-0.51^{ns}	0.59*

^{ns} Non-significant; *,** Significant at 0.05 and 0.01 probability levels, respectively; SL= shoot length; RL= Root length; NR= number of roots,; NS=number of shoots

In this investigation, we found that the genotypes reacted differently to media containing different concentrations of nitrogen and different types of nitrogen. This confirms reports of Evans (1993) and Del Avila *et al.* (1994) that genotypes respond differently to varying nitrogen levels. Also, Powell and Caligari (1989) found that genotypes respond differently to various *in vitro* culture conditions. Some researchers have stated that nitrate alone is not sufficient as a nitrogen source in *in vitro* culture and it is necessary to supplement with a source of reduced nitrogen such as NH_4^+ (Dougall 1981; Timpo and Neyra 1983; Amirouche *et al*. 1985; Del Avila *et al.* 1994). Although some of our findings with NH_4NO_3 are similar to the above reports (N3 level for root length and N2 and N3 levels for the number of roots), some results with KNO_3 (N2 and N3 levels for shoot length, N4 level for root length and N2, N3 and N4 levels for the number of nodes) were different.

It can be concluded that the concentration of the nitrogen source significantly affect both the growth and morphpgenesis characters of potato cultivars in the micropropagation process. In other words, some micropropagation properties and traits which are related to the ability to use different nitrogen concentration in potato are genotype-dependent. Also, the different concentrations of nitrogen sources showed various effects in *in vitro* culture conditions.

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