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SODIUM NITROPRUSSIDE STIMULATES MICROPROPAGATION AND TDZ INDUCES ADVENTITIOUS SHOOTS REGENERATION IN RED FLESH APPLE *MALUS NIEDZWETZKYANA* Koehne Dieck ex

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ABSTRACT

The aim of the present investigation was to optimize protocols for micropropagation and adventitious shoot regeneration from leaf explants of two wild ecotypes of red flesh apple *Malus niedzwetzkyana* for future breeding programs. At the proliferation stage, different concentrations of sodium nitroprusside (SNP) and triacontanol (TRIA) were compared. To optimize shoot regeneration from leaf explants, interactive effects of 1-phenyl-3-(1,2,3-thidiazol-5-yl)-urea – thidiazuron (TDZ), indole-3-butyric acid (IBA) and two explant types were investigated. At rooting stage, the effect of exposure time of microshoots to darkness and exposure time to different concentrations of IBA and α-naphthalene acetic acid (NAA) were compared. The results showed that SNP affected the growth rate significantly and the maximum multiplication rates per explant (9.6 in the first ecotype and 8.8 in the second) were produced in the Quoirin and Lepoivre medium containing 17 SNP μM, in addition to 4 μm 6-benzylaminopurine (BAP) and 3 μm gibberellic acid (GA_3). IBA and TDZ affected the adventitious shoot regeneration from leaf explants significantly, the highest number of regenerated shoots (18.3 per explant) was obtained from the basal section of the leaves cultured on the medium containing 2 μM IBA and 15 μM TDZ. At rooting stage, the maximum rooting (88.6%) was obtained in the result of one weak exposure to darkness on medium containing 3 μM IBA.

Keywords: adventitious regeneration, leaf explants, micropropagation, rooting

INTRODUCTION

Red flesh apple, a wild ecotype, belonging to *Malus niedzwetzkyana* Dieck ex Koehne of Rosaceae family was investigated. This plant has striking red fruit flesh, stems and seeds (Fig. 1), and contains high levels of important phytochemicals – antioxidants, flavonoids and anthocyanins. However, its fruits do not have the quality attributes of today's consumer expectations; thus, application of modern breeding strategies to improve the fruit quality are advantageous. Although in recent years, micropropagation protocols have been developed for some commercial apple cultivars, micropropagation of wild species require specific protocols.

Fig. 1. Seeds and fruit of *Malus niedzwetzkyana*

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Dobránszki and Teixeira da Silva (2010) stated that the success in apple in vitro culture is affected by many factors, including genotype and physiological state of the donor plant, and in vitro conditions (e.g., culture media composition, temperature and light). Studied in our experiment bio-stimulant triacontanol (TRIA) have been identified as natural plant growth regulator that enhances crop production due to involving in several physiological pathways (Naeem et al. 2012). It was reported as a factor increasing dry weight of corn seeds, leaf area of rice, promotion of tobacco callus, stimulation of shoot growth, induction of early flowering, etc. Nitric oxide (NO) is another compound that regulates several physiological processes in plants, including germination, growth, development and plant defense (Baudouin 2011; Wimalasekera et al. 2011). NO promoted growth and development of plants at low concentrations (Beligni & Lamattina 2001). Feng et al. (2013) suggested that NO affect the regulation of cytokinin signaling by reducing phosphorelay activity through S-nitrosylation. Sodium nitroprusside (SNP) applied as nitric oxide (NO) donor was reported as a promotor of proliferation and regeneration in vitro of *Malus hupehensis* Rehd. (Han et al. 2009a).

Thidiazuron – 1-phenyl-3-(1,2,3-thidiazol-5-yl)-urea (TDZ) has been reported to be very efficient for adventitious shoot regeneration in apple (Mitić et al. 2012; Predieri & Malavasi 1989). Cytokinins cannot induce regeneration in the absence of auxins, so appropriate combination of cytokinin and auxins is usually required. The most important auxins include α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA). Seong and Song (2008) recommended 22 μM TDZ and 1.5 μM IBA for adventitious shoot regeneration of 'Fuji' apple. Regeneration response of apple 'Golden Delicious' from primary leaf explants was increased by 70% using modified MS regeneration medium containing two phosphatase inhibitors, 0.9 μM TDZ and 0.5 μM IBA (Grafe & Wricke 1998).

The aim of the present investigation was to evaluate the effect of plant growth regulator compounds including triacontanol and SNP aiming to optimize protocols of micropropagation and adventitious shoot regeneration from leaf explants of two wild ecotypes of Iranian red flesh apple. This is the first inclusive report on tissue culture of red flesh apple, which could be used in future breeding programs in order to breed new cultivars that could satisfy consumer taste.

MATERIALS AND METHODS

Plant material and general procedures

During the early spring, plant tissue cultures of red flesh apple trees *M. niedzwetzkyana* were initiated from donor plants grown in two regions in Iran: Shahrud (Semnan Province) and Aznab (Zanjan Province). Stem cuttings containing lateral buds were divided into pieces with length of 1.5 to 2.5 cm and surface-sterilized according to Jafarkhani Kermani et al. (2009). The explants were placed in the test tubes containing Murashige and Skoog medium (MS) (Murashige & Skoog 1962) supplemented with 2 μM 6-benzylaminopurine (BAP). Throughout the experiments, all the culture media contained 30 $g \cdot dm^{-3}$ sucrose, 100 $mg \cdot dm^{-3}$ phloroglucinol and 6.8 $g \cdot dm^{-3}$ Plant agar (Duchefa, Netherlands) with a pH of 5.8. The media were autoclaved for 15 min at 121 °C and 1.2 kPa. All the explants were incubated in a growth room at 16 h photoperiod under cool-white fluorescent light at a PPFD of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$, at 22 ± 2 °C for 30 days, unless otherwise stated.

Effect of NO and TRIA on micropropagation

Sodium nitroprusside (10, 17, 25, 50, 70 μM) and triacontanol (1, 100, 1000 μM) were included in the medium containing salts and vitamins of Quoirin and Lepoivre (1977) – QL, 4 μm BAP and 3 μm gibberellic acid (GA_3). In all experiments two ecotypes ('Shahroud' and 'Aznab') were used. Each treatment consisted of five jars containing five microshoots with the length of 1.5 to 2 cm. The explants were sub-cultured every 30 days and the growth characteristics, including the number of side shoots, the number of leaves and shoot length (mm) were recorded after two subcultures (60 days).

Adventitious shoot regeneration from leaves

The interactive effect of two PGRs – TDZ (0, 5, 7.5, 10, 15, 20 μM) and IBA (0, 1, 2 μM) – and two explant types (apical and basal leaf sections) were compared. In vitro leaves were cut in the middle and both apical and basal sections were placed face down on the culture media. For each treatment, three Petri dishes containing 10 explants each (5 from apical and 5 from basal section) were used. Petri dishes were incubated in the dark for 4 weeks. The number of shoots per explants and the size of the callus were evaluated 6 weeks later (after 70 days). To record the approximate callus mass, the following system was developed: callus mass diameters up to 2, 4, 6, 8, 10 mm were recoded as callus volume of 1, 2, 3, 4, 5 respectively.

Rooting of shoots

Two experiments were conducted on rooting of 'Shahroud' 3–4 cm long microshoots: 1) on the effect of length of exposure time to darkness and 2) on the effect of concentrations and length of exposure time to two auxins. In the first experiment, microshoots were cultured on ½ strength basal MS medium. The treatments included different lengths (0, 1, 3, 5 and 7 days) of exposure to darkness on auxin-free medium. Each treatment involved three jars with five microshoots. After the initial darkness, the shoots were transferred to the growth room with 16 hour cool-white fluorescent light at a PPFD of 60 μmol m^{-2} s^{-1}. In the second experiment, the elongated shoots were cultured on ½ strength MS media supplemented with either IBA or NAA at four concentrations (0, 1, 2, 3 μM). In each treatment, six jars with five shoots were used. After the initial exposure to one week of complete darkness, half of the explants (three jars) from each treatment were transferred to PGRs free (basal MS) medium and the other half remained in the media containing PGRs. All the explants from all the treatments were moved to the growth room with 16 hour light. After 8 weeks the percent of rooting, number of roots per explant and length of roots per explant (mm) were recorded.

Acclimatization stage

For acclimatization, the gel medium was washed off the roots and the plantlets were transferred into pots with a volume of 200 ml containing perlite, sand and sterilized peat moss in the ratio 1 : 1 : 1. The pots were covered with plastic transparent cups for 4–5 days, then a hole was made in the covering cup every day, until day 10, when the cover was removed completely. This was done to provide 90–100% humidity at the beginning and gradually reducing it to 60%, which was the humidity of the greenhouse. The temperature was kept between 22 and 24 °C. After this period, the explants were transferred to larger pots in a shady place outside the greenhouse.

Statistical analysis

Experiments were in a completely randomized factorial based design. The statistical analysis of the measured characteristics was performed using SPSS (ver. 22). Multivariate variance analysis was applied to determine the significance of differences between treatments. Duncan test ($p < 0.01$ and 0.05) was applied to establish the significance of the differences between each treatment.

RESULTS

Effect of SNP and TRIA on micropropagation

SNP and TRIA affected the growth characteristics significantly. The maximum number of lateral shoots per explant (9.6 in 'Aznab' and 8.8 in 'Shahroud') were observed on the medium containing 17 μM SNP (Fig. 2A). The longest shoots (37 mm in 'Aznab' and 40 mm in 'Shahroud') and the maximum number of regenerated leaves per microshoot (64 in 'Aznab' and 63 in 'Shahroud') were also produced in the same medium (Fig. 2B & 2C). Triacontanol did not influence the number of shoots and leaves, and decreased the shoot length of 'Azrab' (Fig. 2A, 2B, 2C) Moreover, the leaves were larger in 17 μM SNP medium and minute in the medium with 50 and more μM SNP (Fig. 3).

Adventitious shoot regeneration from leaves

All of the leaf explants from 'Aznab' ecotype became necrotic within three weeks and were discarded (Fig. 4A). In all the treatments for both explant types, callus proceeded shoot regeneration and shoots regenerated near the callus mass (Fig. 4B). The differences in callus initiation between the basal and apical sections of the leaves were not significant (Fig. 5A). Callus was not formed in the control treatment without PGRs, but small amounts of IBA or TDZ were sufficient for the initiation of callus (Fig. 5A). Callus growth was stimulated strongly by the addition of both PGRs, especially at higher concentrations (Fig. 5A). On media where more callus was initiated, a higher number of adventitious shoots was observed compared with the media that initiated less callus. There was a significant difference in adventitious shoot regeneration between different types of explants and different concentrations of plant growth regulators (Fig. 5B). The highest number of adventitious shoots were formed on the media containing 2 μM IBA and 7.5, 10 or 15 μM TDZ from basal leaf parts (around 18 per explant; Fig. 5B). The highest number of shoots from apical sections of the leaves (10–12 shoots per explant) was also recorded on the media containing 2 μM IBA and 7.5, 10 or 15 μM TDZ.

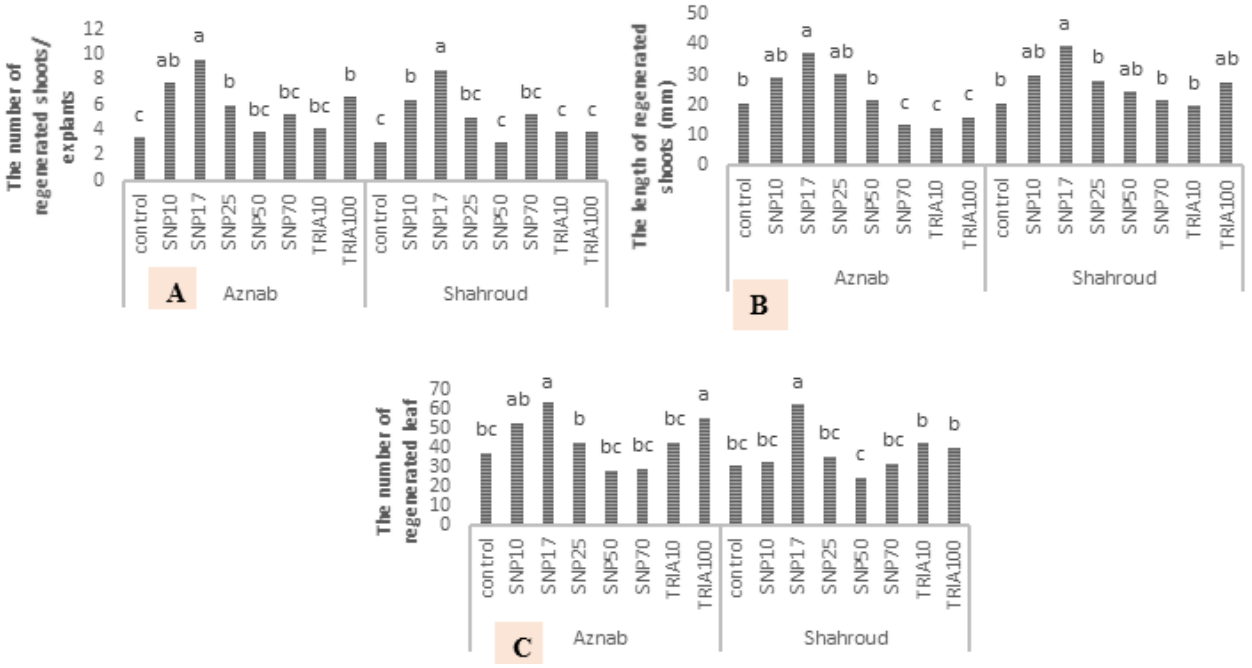

Fig. 2. Effect of concentrations (µM) of SNP and TRIA on shoot multiplication of two ecotypes of *M. niedzwetzkyana* after 8 weeks: A) the number of regenerated shoots, B) the length of regenerated shoots (mm), C) the number of regenerated leaves. Different letters show significant differences according to Duncan's multiple range test ($p \leq 0.01$).

Rooting of shoots

The maximum percent of rooting (58.2%) was obtained on the explants that were incubated for 7 days in darkness on the medium without auxin. However, the differences between treatments in total length of the roots and number of roots per shoot were not significant (Table 1). In experiment 2, one of the highest rooting rate (58.2%) was obtained in the medium without auxin but there only 1.8 roots were formed and they were relatively short (Table 2). In the treatments where shoots were only 1 week on auxin medium, the best rooting was on the medium containing 3 µM IBA (88.6%) followed with 2 µM IBA (55.3%). If the shoots were not transplanted on auxin free medium, the highest percent of rooting was in the media containing 1 µM NAA and 2 µM IBA (55.3%). The most and the longest roots were obtained in the medium with 1 µM NAA and 1 and 2 µM IBA. In general, one week rooting on auxins was more effective compared with four weeks exposure to auxins. Callus was produced on the base of the explants which were rooted on auxin medium for four weeks. The roots obtained in NAA medium were very thin compared with these obtained on the media which contained IBA. The plantlets grown on the media containing IBA were more compact and vigorous compared with the plantlets obtained from media containing NAA.

Acclimatization stage

At acclimatization stage, 10 plantlets from the rooting media containing 3 µM NAA and 3 µM IBA, rooted one week on auxin medium, were compared. The results showed that 100% of the plants rooted on IBA medium acclimated successfully, whereas only 40% of these rooted on NAA medium were able to acclimatize in greenhouse conditions.

Fig. 3. Influence of three concentrations of SNP on size of leaves

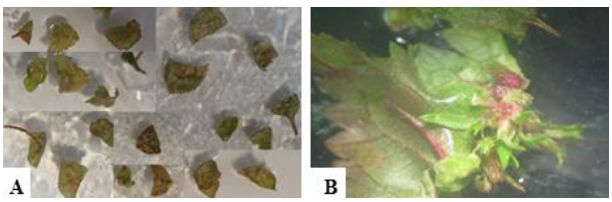

Fig. 4. A) necrosis of the leaf explants of 'Aznab' ecotype; B) regeneration associated with callus formation in apical section of leaf explant of red flesh apple 'Shahroud' ecotype.

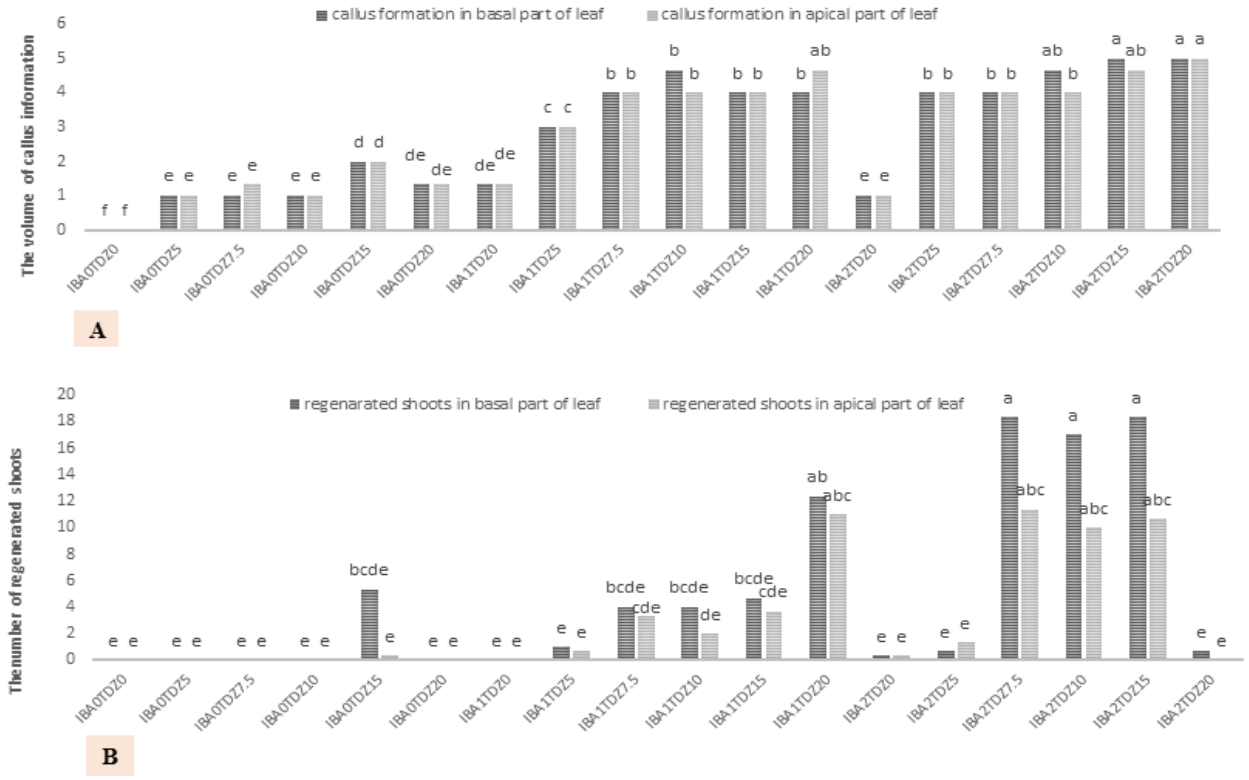

Fig. 5. Effect of IBA and TDZ and explant type (basal and apical sections of leaf) on A) callus formation and B) adventitious shoot regeneration of red flesh apple 'Shahroud'. Different letters show significant differences according to Duncan's multiple range test ($p \leq 0.01$).

Table 1. The effect of number of days in darkness on rooting percentage, total length of the roots (mm) and the number of roots per shoot in 'Shahroud' ecotype

Number of days in darkness	Rooting percent	Total length of roots (mm)	The number of roots
0	27.1 b	9.3 a	2.3 a
1	24.3 b	3.2 b	2 a
3	26.7 b	11.7 a	2.7 a
5	37.2 b	10 a	2.7 a
7	58.2 a	7.9 a	1.8 a

Different letters at each column, show significant differences according to Duncan's multiple range test ($p \leq 0.05$).

Table 2. Effect of different concentrations of two auxins with two treatment time on rooting percent, total length of the roots (mm) and the number of root/shoot in 'Shahroud' ecotype

Period of auxin treatment	Type of auxin	Concentration of auxin (µM)	Rooting (%)	Total length of roots (mm)	The number of roots
	control	0	58.2 ^{ab}	7.9 ^{c}	1.8 ^{b}
1 week	NAA	1	33.3 ^{b}	**45.4** ^{a}	**4.9** ^{a}
		2	44.3 ^{b}	18.6 ^{b}	1.3 ^{b}
		3	25.6 ^{c}	21.7 ^{b}	0.4 ^{c}
	IBA	1	33.3 ^{b}	35.7 ^{ab}	0.8 ^{c}
		2	55.3 ^{ab}	18.9 ^{b}	1.1 ^{b}
		3	**88.6** ^{a}	14.8 ^{bc}	2.2 ^{ab}
4 weeks	NAA	1	55.3 ^{ab}	14.0 ^{bc}	4.6 ^{a}
		2	44.3 ^{b}	4.7 ^{c}	3.0 ^{ab}
		3	22.0 ^{c}	1.27 ^{c}	0.2 ^{c}
	IBA	1	44.3 ^{b}	24.6 ^{b}	1.1 ^{b}
		2	55.3 ^{ab}	13.4 ^{bc}	2.4 ^{ab}
		3	25.6 ^{c}	10.5 ^{bc}	0.6 ^{c}

*Note: see Table 1

DISCUSSION

In the present study, micropropagation effectivity of both ecotypes of. *M. niedzwetzkyana* was increased significantly as a result of SNP addition, similarly as in the experiments with *Malus hupehensis* (Han et al. 2009a). The mechanism by which SNP as an NO donor improves the micropropagation efficiency is still not very clear. However, several studies have shown that NO interacts with different PGRs (auxins, cytokinins, gibberellins, ethylene) at different steps of signaling pathways to evoke various responses what finally increases the growth rate (García-Mata & Lamattina 2002). Carimi et al. (2005) found that cytokinin BAP induced NO accumulation in cell suspension cultures. Ötvös et al. (2005) suggested that NO may interact with auxin and cytokinin, linking the regulation of cell division with differentiation during de-differentiation and re-differentiation of plant cells. Tan et al. (2013) showed that nodal segments of *Vanilla planifolia* produced higher number of shoots on medium containing 1.0 $mg \cdot dm^{-3}$ BAP and 10 μM SNP, compared with the medium supplemented only with BAP. Beligni and Lamattina (2000) reported that NO affects plant developmental events in which gibberellins are essential in order for them to operate. Although many reports imply that SNP has positive effects on shoot proliferation of in vitro plantlets, Sarropoulou et al. (2015) reported that combination of SNP and BAP reduced in vitro shoot proliferation of cherry rootstock. Verma et al. (2014) also reported that, when SNP was used at a concentration of 500 μM, proliferation of plantlets declined and leaves turned brown and necrotized in two peanut cultivars. Triacontanol, a natural component of the epicuticular waxes has also been shown to increase the vegetative growth. Shams El-Din (2018) investigated the effect of TRIA on rooting of 'Sewi' date palm and showed that medium containing 5 $\mu g \cdot dm^{-3}$ TTRIA and 1 $mg \cdot dm^{-3}$ GA_3, produced the highest number and longest roots and highest shoots compared with the media without TRIA. In the present investigation, TRIA had a significant effect only on the number of regenerated leaves in one ecotype and was not as effective as SNP for shoot multiplication and growth of red flesh apple.

Callus production is an important step in the regeneration of adventitious shoots. In the present study, calli appeared mainly on the wounded edges and midribs of the leaf explants and regenerated shoots developed near the formed callus. Association of callus formation and organogenesis has also been reported by Hatamian et al. (2014) in quince. Chitra et al. (2017) compared protein profiles in mulberry leaves during different stages of callus and shoot induction and showed a decrease in 49 kDa protein in leaf explants during callus proliferation and shoot formation, whilst 39 kDa was increased when shoot organogenesis occurred. They concluded that the change in proteins illustrate the expression of genes related to leaf organogenesis. Therefore, higher rates of shoot regeneration from the basal section of the leaves in the present study could be attributed to the physiological events, which may have taken place there. Fasolo et al. (1989) showed that for induction of shoot regeneration in apple leaf explants, a high cytokinin to low auxin ratio is essential, which also resulted from our experiment. Zhang et al. (2014) reported that organogenic potential of apple leaf explants was directly related to the concentration of TDZ and NAA in the culture medium. They showed nearly complete (99%) shoot formation on medium containing 2.7 μM TDZ and 0.9 μM NAA. Jin et al. (2014) showed that proper combination of TDZ with IBA resulted in 100% regeneration, but increase in TDZ concentration decreased regeneration potential. This is in accordance with the results of the present investigation where the number of regenerated shoots significantly decreased on the medium containing 20 μM TDZ. The ability of TDZ to stimulate shoot regeneration has been reported in other plant species, including *Prunus* (Liu & Pijut 2008), pear (Javadi et al. 2013), peach (Soliman 2013), and rose (Pourhosseini et al. 2013).

Han et al. (2009b) in a review illustrated that root initiation during cell de-differentiation is associated with a presence of auxins, however, rooting percentage is related to the duration of explants' exposure to auxin. At rooting stage, the tissue at the base of the explant first becomes competent, i.e., capable of reacting to the auxin, and then, with the following prolonged culture on media containing auxin, the tissue is driven to the process of root formation (Meins & Binns 1979; Christianson & Warnick 1983). Most woody plants require a sequence of two rooting media. A high auxin concentration is only required during the root initiation, but root development and emergence is inhibited by high concentrations of auxins (Klerk et al. 1990).

CONCLUSION

Regeneration of adventitious shoots was most efficient on the basal parts of leaves on the MS media containing 2 μM IBA and either 7.5, 10 or 15 μM TDZ. Shoot multiplication was the highest in the MS medium containing 2 μM BAP and 17 μM SNP. The above results can be used in breeding purposes and in mass propagation and commercialization of *Malus niedzwetzkyana*.

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