



Optimized Root Production During Micropropagation of New Iranian Apple Hybrid Rootstock (AZ X M9): Effects of Fe-EDDHA and Thiamine

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Abstract

In vitro propagation of AZ x M9 new apple rootstock resulted of breeding program of vegetative apple rootstock in Iran was investigated. Nodal explants were inoculated in Murashige and SKoog (MS) medium supplemented with various concentrations of 6-benzylaminon purine (BAP) (0.4, 0.8 and 1.2 mg/l) alone or with naphthalene acetic acid (NAA) (0.1 mg/l). We examined the effect of different concentrations of iron sequestrene (Fe-EDDHA) and thiamin vitamin in ½ MS and Linsmaier and Skoog (LS) media supplemented with indol-3-butric acid (IBA) (1 mg/l) on rooting of attained plantlets. According to our results, 0.4 BAP + 0.1 NAA treatment in MS medium was the best component which resulted in the highest proliferation rate (6.73). As well, we estimated the highest root number in ½ MS + 0.15 g/l Fe-EDDHA + 2.4 g/l thiamin.

Key words: Iranian apple vegetative new hybrid rootstock, *In vitro* culture, Shoot regeneration, Rooting

Introduction

Apple is the third most important fruit crop (64.3 million t/year) in the world (FAO, 2009). Apple is conventionally propagated by budding or grafting. In order to developing apple vegetative rootstocks compatible with Iran apple cultivation areas soils, some targeted hybridization was carried out between Iranian dwarf genotypes and commercial vegetative rootstock (M9) as maternal and paternal parents, respectively, in Seed and Plant Improvement Institute, Karaj, Tehran, during 2005–2007. There were many rooting power (main and adventitious root number) differences among various obtained offspring which is of high importance in suitable rootstock establishment in soil and dryness stress resistance. So that, offspring related to Isfahan Azayesh as maternal parent showed most appropriate rooting.

Traditional propagation methods depend on the season and typically result in low multiplication rates. Micropropagation methods provide an efficient and alternative way for the commercial propagation of

plants and among fruits, *in vitro* propagation of apple rootstocks has been reported by many authors (Minaev *et al.*, 2003; Kaushal *et al.*, 2005; Dalal *et al.*, 2006; Ciccotti *et al.*, 2008). Furthermore, micropropagation allows quick propagation of new varieties or breeding lines or variants for apple breeders. It is an essential step in the success of regeneration of transgenic lines and determines the effectiveness of a transformation protocol (Aldwinckle and Malnoy, 2009). The present study aimed to investigate micropropagation ability of mentioned new hybrid apple rootstock.

Materials and methods

Samples were collected of seedlings established in Horticultural Research Center (Seed and Plant Improvement Institute) during June 2011. Axillary and apical buds of current year shoots (1-2.5 cm) were used as explants. Explants inoculated in MS (Murashige and Skoog, 1962) medium supplemented with concentrations of BAP (6-benzylaminon purine) (0.4, 0.8 and 1.2 mg/l) alone or with NAA (naphthalene acetic acid) (0.1 mg/l) after disinfection. 0.1 mg/l citric acid applied during disinfection to prevention of phenol exudation. Proliferation rate of established explants was determined after 30 days by estimating the number and length of regenerated axillary shoots. Two culture media of LS (Linsmaier and Skoog, 1965) and MS supplemented with 1 mg/l IBA (Indol-3-butiric acid), 0.1, 0.15 and 0.2 g/l Fe-EDDHA and 0, 0.8, 1.6 and 2.4 g/l thiamin. After 30 days root number, length and fresh and dry weights were determined. 7.5 g/l agar and 30 g/l sucrose was used in all media and their pH was adjusted to 5.7.

Statistical analysis

Experiments were performed as completely randomized design (CRD) with 5 replications and 3 explants for proliferation and 2 explants for rooting tests. SPSS software version 12 was used for data analysis. Means were compared by Duncan's Multiple Range Test ($p < 0.05$).

Results and discussion

Shoot multiplication

The success and commercial usefulness of an apple micropropagation protocol largely depends on the mode and rate of shoot multiplication. The efficacy of shoot multiplication is influenced by several factors, such as media composition, plant growth regulators, etc. (Dobránszki *et al.*, 2010). According to fig. 1a, the highest shoot number (6.73) was observed in treatment 0.4 mg/l BAP + 0.1 mg/l NAA with insignificant difference toward 1.2 mg/l BAP + 0.1 mg/l NAA. The lowest shoot number (1.26) was observed in 0.4 mg/l BAP supplemented media which was insignificantly different with 0.8 mg/l BAP. It can be received that probably there is a positive relationship between shoot number and existing NAA in culture medium. As well, the lowest amount of BAP along with NAA showed the highest shoot number. The highest shoot length (3.0 cm) was estimated in the media containing 0.8 mg/l BAP which was insignificantly different to 0.4 mg/l BAP + 0.1 mg/l NAA treatment. The lowest shoot length (1.03 cm) was observed in 0.4 mg/l BAP. Low proliferation rate of 0.8 mg/l BAP treatment (fig. 1b) confirms Marin *et al.* (1993) theory which describes that there is negative relationship between shoot number and length. Shoot branching depends on the initiation and activity of axillary meristems, which are hormonally controlled mainly by cytokinins; however, they act in interaction with auxins even though the auxin-effect is indirect (Ward and Leyser, 2004). Shoot multiplication of apple is based on media containing cytokinins as the major PGR, in lower concentration also auxins and the effect of different PGRs is highly genotype dependent (Dobránszki *et al.*, 2010).

Butiuc-Keul *et al.* (2010) found that shoot production increased in apple cultivars in response to BAP in culture medium. They showed that 1.0 mg/l BAP is adequate for shoot multiplication. But they obtained the highest length of shoots in media with 0.5 mg/l BAP irrespective of apple cultivar. Superiority of BAP in inducing axillary branching or shoot bud differentiation has been reported particularly in case of woody plants (Murashige, 1974; Chaturvedi *et al.*, 2004). Soni *et al.* (2011) found BAP the most effective cytokinin in shoot multiplication, and a relationship between BAP concentration, shoot number and shoot size. They detected that higher concentration of BAP alone may increase the shoot number while decreasing shoot length whereas low concentrations led to longer shoots. They also found that BAP (0.5 mg/l) combined with IBA (0.01 mg/l) increased the rate of shoot production as well as length of shoots. It has been revealed in many other apple rootstocks (Miri *et al.*, 2003; Zhang *et al.*, 2004; Kaushal *et al.*, 2005). Zimmerman (1984) has suggested that differential sensitivity to a particular cytokinin and auxin, to the concentration of each, and to the ratio between them are important factors, especially in the establishment and proliferation of cultures.

Rooting

Successful rooting of *in vitro* shoots prior to their establishment in soil is a prerequisite for any propagation method (Dobránszki *et al.*, 2010). It was found that LS medium was not useful for rooting as it just resulted in callusing. $\frac{1}{2}$ MS medium containing 0.1 g/l used iron and all concentrations of thiamine just also resulted in callusing. As it is observed in fig. 2 a, the interaction of thiamin and Fe-EDDHA was significant on root number. The highest number of root (3.5) was found in 2.4 g/l thiamin + 0.15 g/l Fe-EDDHA. The lowest number of root (0.75) was detected in 0.8 g/l thiamin + 0.15 g/l Fe-EDDHA with insignificant difference toward 0.2 g/l Fe-EDDHA. Significant interaction of Fe-EDDHA and thiamin on root length is evident in table 2. The highest root length (3.25 cm) was detected in 0.2 g/l Fe-EDDHA + 0.8 g/l thiamin and 0.15 g/l Fe-EDDHA + 2.4 g/l treatments. The lowest root length (0.784 cm) was related to treatment containing 0.15 mg/l Fe-EDDHA which was significantly lower than the rest treatments (fig. 2 b). It can be realized of fig. 2 b results that increase in Fe-EDDHA and thiamin concentrations negatively affect each other so that in 0.15 g/l Fe-EDDHA + all concentrations of thiamin, root length enhancement was occurred while the lowest root length increase was obtained in 0.2 g/l Fe-EDDHA without thiamin and root length decreased slowly in 0.8, 1.6 and 2.4 g/l thiamin. It was realized that Fe-EDDHA caused more qualitative roots (thin and flexible) which were more suitable for hardening. *In vitro* plantlets transferred directly to pot mix (perlite and coco-peat 1:1 v/v strengthened with $\frac{1}{2}$ MS).

To our knowledge, this is the first report of using Fe-EDDHA and thiamin on *in vitro* rooting of apple but in other plant species like GF677 Sepahvand *et al.* (2012) indicated that the lowest percent of rooting occurred in LS medium without thiamine and the highest rooting percentages were obtained in LS medium with thiamine at 1.6 mg/l, IBA at 1, 1.3 and 1.6 mg/l or thiamine at 2.8 mg/l, IBA at 1.3 and 1.6 mg/l. Thiamine at 1.6 and 2.8 mg/l produced the highest number of roots. However, its application at 4 mg/l or even control had the lowest number of roots. Application of thiamine in LS medium had a great effect on root number and length, but increasing its amount to 4 mg/l had reductive effect on root number and there were no significant effect between thiamine at 4 mg/l and control. Thiamine as an enzyme cofactor has important effect on metabolically reactions such as glycolysis or in pentose phosphate and tricarboxylic acid cycle. In addition to thiamine value as a nutritional compound, it is also secondary messenger in activation of proteins with low molecular weight. Thiamine also causes increment in expression of genes involved in producing defective enzymes which increase plant resistance to plant pathogens (Hwan lee and Soonok, 2005). In contrast to our research, earlier apple rootstocks reports are on the effects of phytohormones like IBA on rooting (Castelli *et al.*, 1986; Soni *et al.*, 2011). Plantlets

were washed and soaked in benomyl fungicide to reduce incidence of disease and wilting. Protecting plants from dehydration during the first week of *ex vitro* growth was crucial for successful hardening. Foliar feeding of plants with mineral salts improved vigor of plants. Some plants were hardened and were transferred out of the greenhouse (data not shown). In conclusion, we suggest a micropropagation method in AZ x M9 new apple hybrid rootstock. Because the proliferation rate is the main consideration for successful large-scale plant production, further investigations are in progress to achieve *in vitro* propagation commercialization of this hybrid and other progenies obtained from this hybridization process, comparing their *in vitro* reaction.

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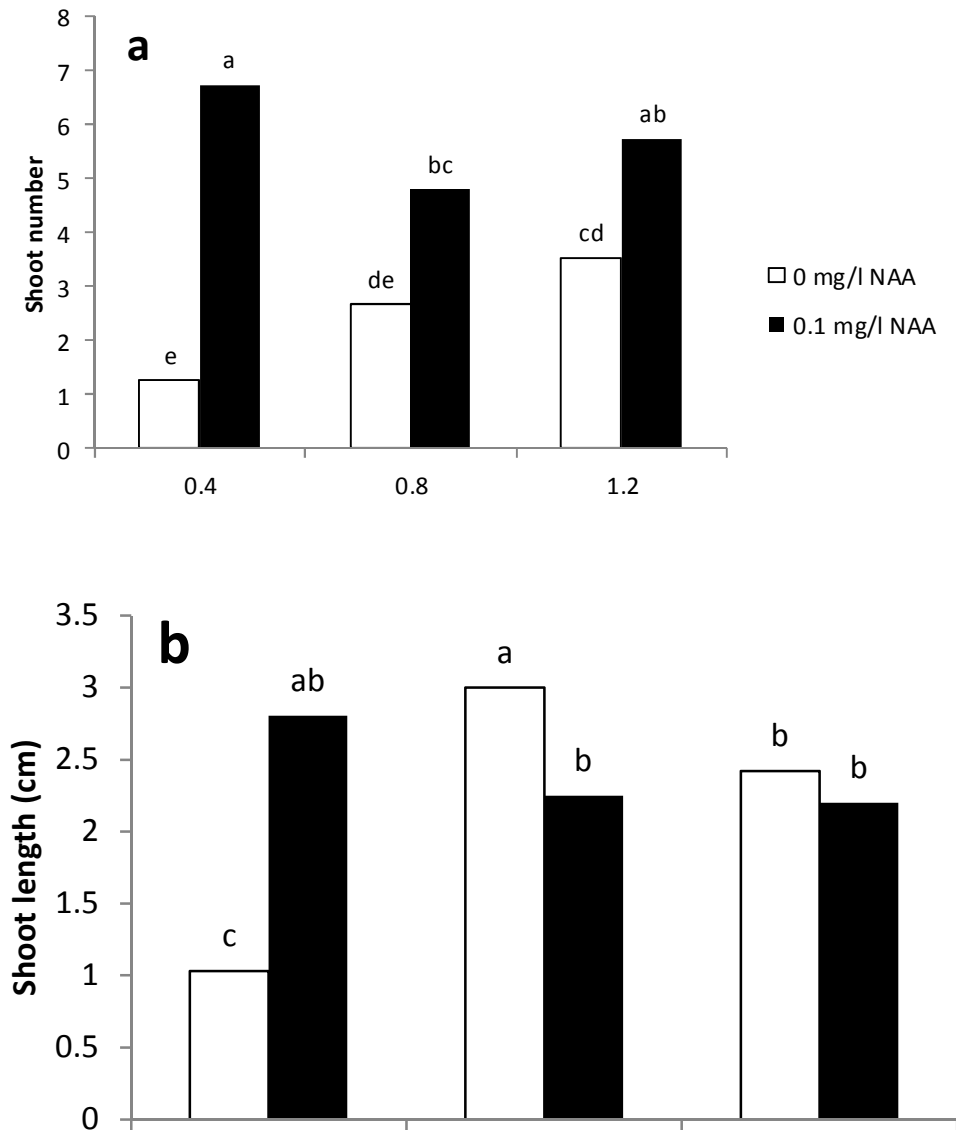


Fig. 1. Interaction of BAP and NAA concentrations on *in vitro* proliferation of AZ x M9 apple rootstock.

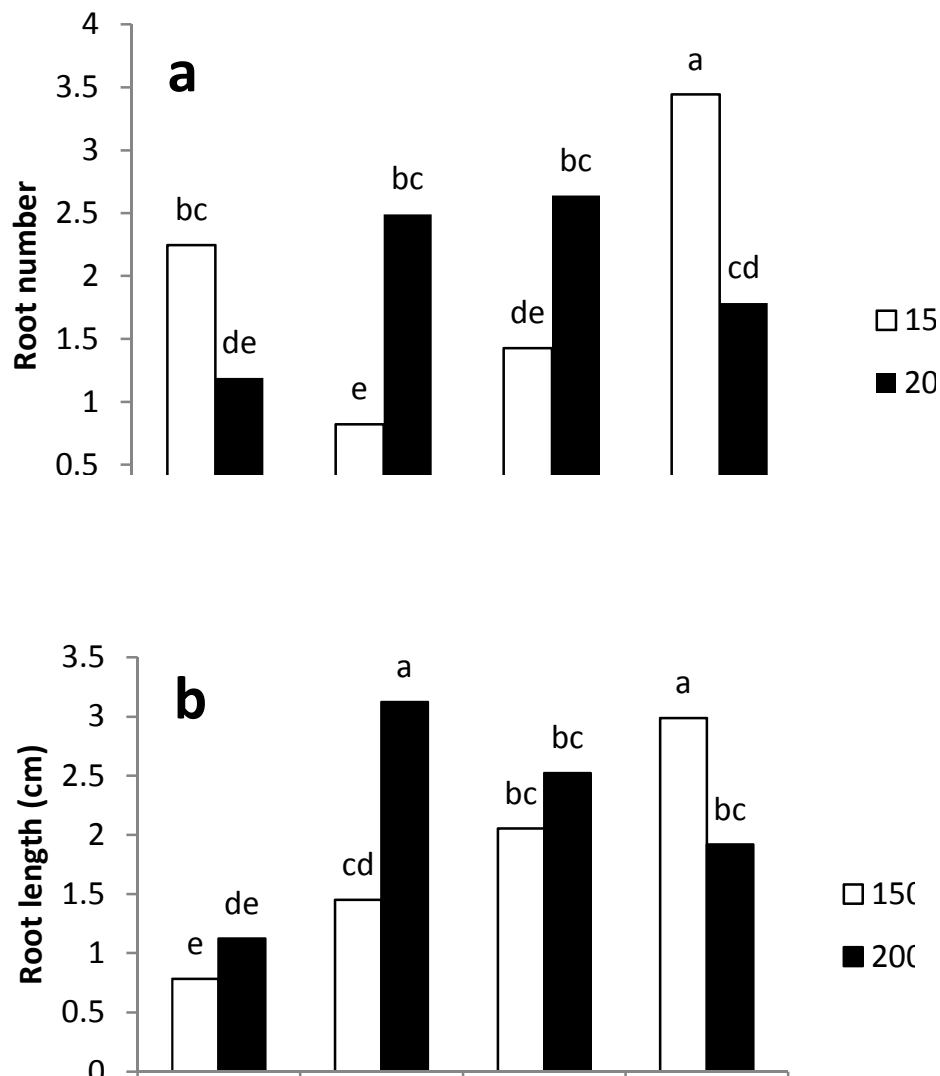


Fig. 2. Interaction of Fe-EDDHA and thiamin concentration on *in vitro* rooting of AZ x M9 apple rootstock.