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L-GLUTATHIONE-REDUCED ENHANCES IN VITRO ROOTING OF APPLE ROOTSTOCK MM106 (*Malus domestica* Borkh.)

SUMMARY

The experiment was carried out to study the effect of L-glutathione-reduced (GSH) on rooting, vegetative growth and callusing under *in vitro* conditions of shoot tip explants in MM106 apple rootstock. GSH was applied at 10 concentrations (0-1000 μM) in combination with 5.4 μM α-napthaleneacetic acid (NAA). After 28 weeks of culture in the rooting media, obtained results indicated that the incorporation of 10 μM GSH into the MS culture medium resulted in the maximum root number per rooted explant (11.67), greatest root length (41.11 mm) and root fresh weight (FW) (0.289 g). However, the highest rooting percentage (50%) was achieved in the 25 μM GSH + 5.4 μM NAA combination treatment. The results also cleared significant increase in shoot length (41.43 mm) and shoot FW (0.232 g) by 2.81 cm and 3.5 times, respectively compared with the control GSH-untreated explants. Callus FW (0.699 g) and callusing percentage (57.14%) were maximum by adding 10 μM GSH to the medium. Neither rooting nor callusing was recorded in the absence of GSH. In addition, the application of 5, 50 or 1000 μM GSH resulted in complete inhibition of rooting. No callus induction occurred when the explants were treated with 5, 100, 250 or 1000 μM GSH. Taking into consideration all the aforementioned it is clearly demonstrated that 10 μM GSH when applied simultaneously with 5.4 μM NAA enhanced rooting and shoot growth of microcuttings in a considerable degree. Therefore, GSH can be used as a rooting promoting agent in MM106 apple rootstock tissue culture system.

Keywords: antioxidants, apple rootstock, *in vitro* rooting, L-glutathione, *Malus domestica* Borkh, thiol compounds.

INTRODUCTION

Apple (*Malus domestica* Borkh.) is one of the most important fruits in temperate-zones. It is the third most important fruit tree in the world (FAO 2013). Apple is conventionally propagated by vegetative methods, such as budding or grafting. Although these traditional propagation methods do not ensure disease-free and healthy plants, they depend on the season; moreover, they typically result in low multiplication rates. Micropropagation provides the rapid propagation of new varieties, breeding lines or mutants in apple breeding

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because it is the most necessary stage in the regeneration of transgenic lines and determines the effectiveness of a transformation protocol (Aldwinckle and Malnoy 2009). Recently in apple, many reliable methods have been developed for both propagation of rootstocks and scions using *in vitro* techniques. Successful micropropagation of apple using microcuttings or single node cuttings is influenced by several internal and external factors, including genotype, physiological state of sampling, *in vitro* media constituents and their ratio, light, temperature and other factors (Dobránszki and Silva 2010). Micropropagation of apple rootstocks depends upon new areas of research and fruit tree propagation allowing the problems of conventional methods to be overcome and enabling rapid multiplication of disease-free fruit plants at a commercial scale (Zhu *et al.* 2005). Micropropagation of apple to produce self-rooted plants will open up new areas of research and will allow changes in traditional fruit tree propagation (Modgil *et al.* 1999). Micropropagation of apple rootstocks has opened up new areas of research and fruit tree propagation allowing the problems of conventional methods to be overcome and enabling rapid multiplication of disease-free fruit plants at a commercial scale **(**Bahmani *et al.* 2009). So far, apple micropropagation has been attempted with only varying success (Marin *et al.* 1993).

Clonal apple rootstocks have been widely used in apple growing. Among these M9 (dwarf), MM106 (semi-dwarf), and MM111 (semi-vigorous) have been well known and used in various types of soils and plantation systems. These rootstocks have been propagated by stool bed layering and rooting of hardwood cuttings. The MM106 and MM111 apple rootstocks can be rooted easily by hardwood cuttings (Hartmann and Kester 1983). MM106 is a clone of apple that has been originated as a cross between Northern spy and Malling 1 (Hartmann *et al.* 2002). Trees on MM106 are well anchored, do not sucker, are semi-dwarfing (60 - 75% the size of trees on apple seedlings), and very productive (Wiley, 1987).

Low molecular weight antioxidants, such as ascorbate, glutathione, and tocopherol, are information-rich redox buffers that interact with numerous cellular components. In addition to crucial roles in defence and as enzyme cofactors, cellular antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and death (Potters *et al.* 2004; Tokunaga *et al.* 2005). Localized activity of glutathione could also help elucidate the mechanism of stress resistance. This effect indicates that glutathione may be involved in protection against DNA damage (Lodhi, 1998). Glutathione is a small, ubiquitous molecule that is involved in a plethora of cellular processes in addition to its role as an antioxidant and in the maintenance of cellular redox homeostasis (Schafer and Buettner 2001).

Glutathione is crucial for biotic and abiotic stress management as is a pivotal component of the glutathione-ascorbate cycle, a system that reduces poisonous hydrogen peroxide (Noctor and Foyer 1998). It is the precursor of phytochelatins, side-chain and an antioxidant, preventing damage to important

cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella *et al.* 2003). Glutathione is found almost exclusively in its reduced form, since the enzyme reverting it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced glutathione to oxidized glutathione (Anna *et al.* 2003) within cells is often used scientifically as a measure of cellular toxicity. Some scientists suggest that rooting of micropropagated plants can be improved by treatment with antioxidants (Stonier, 1971). Antioxidants can potentially protect the natural plant rooting hormones from oxidation, enhancing rooting and increasing the tolerance of plants to greenhouse conditions (Lis-Balchin, 1989).

The aim of the current research was to study the influence of the thiol compound GSH in a wide range of concentrations to induce rooting of MM106 apple rootstock microshoots in combination with the auxin NAA (α-napthaleneacetic acid), as an auxin source. The main goal of this research study was to verify if and up to what extent the combined effect of GSH+NAA exhibits better rooting results than the individual application of NAA under *in vitro* conditions.

MATERIAL AND METHODS

Plant material and culture conditions

The experimental plant material was shoot tip explants from previous *in vitro* cultures provided by the Plant Tissue Culture Laboratory "VITRO HELLAS S.A., Tree and Plant Nurseries, Alexandria, Nisseli Imathias, Greece". The initial material was certified as virus-free. The nutrient medium used was the MS (Murashige and Skoog 1962). The effects of GSH applied exogenously at ten concentrations (0, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 μM) in combination with 5.4 μM NAA were studied in order to enhance root regeneration of the apple rootstock MM106 (*Malus domestica* Borkh.) under *in vitro* conditions. The explants were grown in glass flat bottom test tubes (25×100 mm) containing 10 ml of MS medium. The culture medium was also supplemented with 30 g/l sucrose and 6 g/l agar (Bacto-Agar; Voigt Global Distribution Inc., Lawrence, USA). The pH of the culture medium was adjusted to 5.8 before adding agar and then the medium was sterilised by autoclaving at 121 °C for 20 minutes. One explant was aseptically transferred to each test tube which was capped with aluminium foil. All the cultures were incubated in a growth room under controlled environmental conditions i.e. a 150 μmol/m^2/s light intensity provided by cool white fluorescent lamps (36W, Philips), a 16 h photoperiod and a 22 ± 1 °C temperature. Mean root number per rooted explant, root length, root fresh weight (FW), rooting percentage, shoot length and shoot FW, callus FW and callus induction percentage were recorded after 28 weeks of explants' maintenance in the rooting media in order to obtain full response.

Statistical analysis

The experimental layout was completely randomised and the data were analysed with ANOVA (Analysis of Variance) using the statistical package SPSS

17.0 (SPSS Inc, Chicago, USA). The experiment was repeated twice and the reported data are the means. The experiment consisted of 10 treatments each one with a total of 10 replicates. To establish significant differences among the treatments, the Duncan's multiple range test was used at $P \leq 0.05$ for mean comparison.

RESULTS AND DISCUSSION

In the control treatment, neither rooting nor callus induction was observed. However, the incorporation of 2.5-500 μM GSH led to root formation (Figure 1a-1e) while GSH applied at 5, 50 or 1000 μM concentrations completely inhibited rooting. Better rooting results in terms of root number/rooted explant (11.67), root length (41.11 mm) and root FW (0.289 g) were achieved with 10 μM GSH. However, 25 μM GSH exhibited the highest rooting percentage (50%) (Table 1).

Table 1. Effect of L-glutathione reduced (GSH) concentration (0-1000 μM) combined with 5.4 μM NAA (a-naphthaleneacetic acid) on average root number/rooted microcutting, root length (mm), root FW (g) and rooting percentage (%) in MM106 apple rootstock.

GSH (μM)	Root number/rooted microcutting	Root length (mm)	Root FW (g)	Rooting (%)
0	0.00 ± 0.00 a	0.00 ± 0.00 a	0.000 ± 0.000 a	0 a
2.5	5.00 ± 0.45 cd	30.63 ± 0.47 e	0.278 ± 0.012 d	28.57 b
5	0.00 ± 0.00 a	0.00 ± 0.00 a	0.000 ± 0.000 a	0 a
10	**11.67 ± 0.34 e**	**41.11 ± 0.91 g**	**0.289 ± 0.031 d**	42.86 c
25	5.50 ± 0.52 d	13.20 ± 0.85 c	0.083 ± 0.012 b	**50 d**
50	0.00 ± 0.00 a	0.00 ± 0.00 a	0.000 ± 0.000 a	0 a
100	5.00 ± 0.00 cd	5.00 ± 0.00 b	0.005 ± 0.000 a	25 b
250	2.50 ± 0.07 b	19.58 ± 1.80 d	0.060 ± 0.007 b	22.22 b
500	4.50 ± 0.37 c	37.50 ± 0.37 f	0.163 ± 0.014 c	22.22 b
1000	0.00 ± 0.00 a	0.00 ± 0.00 a	0.000 ± 0.000 a	0 a
P-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ***$P \leq 0.001$

Shoot length was augmented by 2.81 cm (from 13.33 mm in the control to 41.43 mm) and a 3-fold increase in shoot FW (from 0.064 g in the control to 0.232 g) was recorded by adding 10 μM GSH to the enriched with 5.4 μM NAA MS medium. Callusing percentage was highest (57.14%) and callus FW (0.699 g) greatest when the explants were treated with 10 μM GSH. The incorporation of 2.5-500 μM GSH led to callus formation while GSH when applied at 5, 50 or 1000 μM concentrations resulted in complete inhibition of callusing (Table 2).

Rooting is affected by numerous endogenous and exogenous factors, with the principal role of auxin as a chief regulator of adventitious root formation.

Blocking the transport of endogenous auxin to seedling rooting zone inhibits rooting (De Klerk *et al.* 1999). In the regulation of adventitious rooting process, glutathione seems to be involved in a complex interplay between auxin and other components of cellular redox systems.

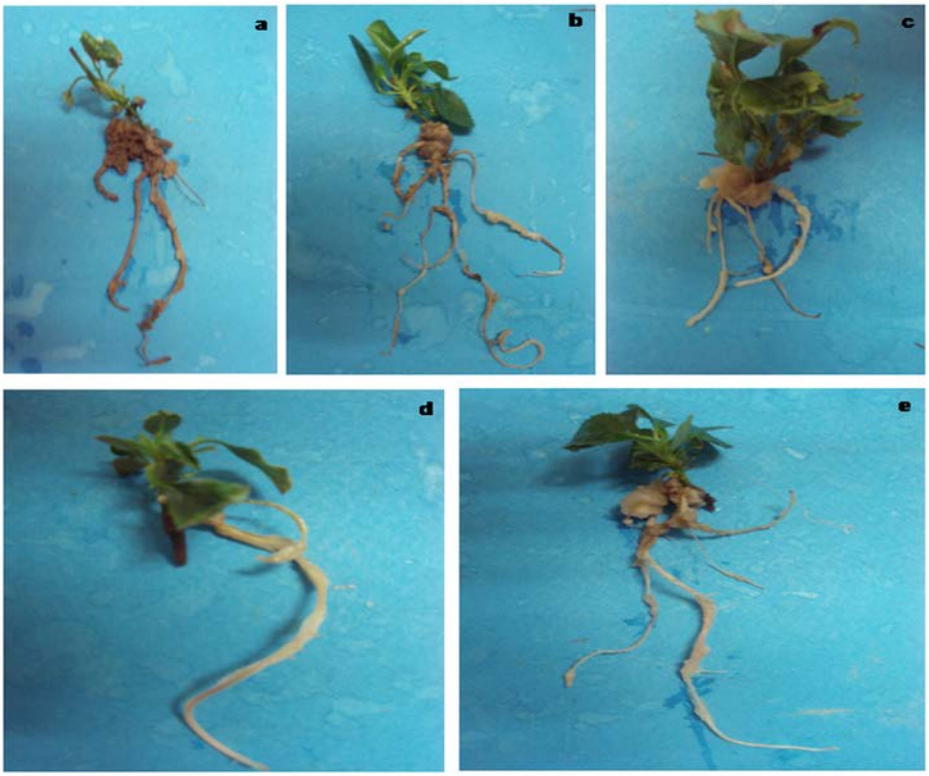

Figure 1. Effect of GSH (0-1000 μM) concentration combined with 5.4 μM NAA on *in vitro* rooting of shoot tip explants of MM106 apple rootstock: (a) 2.5 μM GSH, (b) 10 μM GSH, (c) 25 μM GSH, (d) 250 μM GSH and (e) 500 μM GSH.

In the present study employing the MM106 apple rootstock, GSH in the presence of NAA auxin significantly promoted rooting of shoot tip explants. In accordance with our findings, root formation in shoot cuttings of soybean (*Glycine max* L. 'Williams'), mungbean (*Phaseolus aureas* Mdlbg.), English ivy (*Hedera helix* L.), and apple *(Malus* x *domestica* Borkh. 'Jork 9') was stimulated by GSH in the presence and absence of auxin (IAA: indole-3-acetic acid) shock (Auderset *et al.* 1996). However, no root regeneration in MM106 apple vitroplants occurred in the absence of GSH from the MS containing NAA medium. According to Auderset *et al.* (1996), root number was positively influenced when 100 μM GSH were used simultaneously with IAA in both soybean and mungbean (*P. aureas* Mdlbg.) cuttings. The inclusion of 10 μM GSH to the medium gave the best results regarding root number and root length of MM106 apple microshoots while 25 μM GSH led to the highest rooting

percentage. Similar results were obtained by Auderset *et al.* (1996) in micropropagated apple *(Malus* x *domestica* Borkh. 'Jork 9') shoots derived from callus where 25-100 μM GSH augmented the percentage of rooted explants while root number was increased in the 50-75 μM GSH concentration range. Accordingly, *in vitro* rooting of sweet cherry *(Prunus avium* L.) cv. 'Kristiina (root number and rooting percentage) was significantly promoted by fortifying the MS containing 9.84 μM IBA medium with 25 μM GSH (Vasar, 2004).

Table 2. Effect of L-glutathione reduced (GSH) concentration (0-1000 μM) combined with 5.4 μM NAA (a-naphthaleneacetic acid) on average shoot length (mm), shoot FW (g), callus FW (g) and callus induction percentage (%) in MM106 apple rootstock.

GSH (μM)	Shoot length (mm)	Shoot FW (g)	Callus FW (g)	Callus induction (%)
0	13.33 ± 0.43 a	0.064 ± 0.004 ab	0.000 ± 0.000 a	0 a
2.5	30.00 ± 1.97 e	0.136 ± 0.013 cd	0.431 ± 0.049 c	57.14 d
5	28.00 ± 0.94 de	0.154 ± 0.006 a	0.000 ± 0.000 a	0 a
10	**41.43 ± 4.76 f**	**0.232 ± 0.047 e**	0.699 ± 0.102 e	57.14 d
25	25.00 ± 2.36 cde	0.196 ± 0.028 d	0.149 ± 0.009 b	50 c
50	13.75 ± 0.87 ab	0.073 ± 0.007 a	0.570 ± 0.000 d	25 b
100	20.00 ± 1.29 bc	0.091 ± 0.010 cd	0.000 ± 0.000 a	0 a
250	23.33 ± 2.11 cd	0.121 ± 0.018 b	0.000 ± 0.000 a	0 a
500	20.00 ± 1.29 bc	0.114 ± 0.019 c	0.239 ± 0.013 b	22.22 b
1000	14.44 ± 1.38 ab	0.037 ± 0.006 a	0.000 ± 0.000 a	0 a
P-values	0.000***	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$, ***$P \leq 0.001$.

The combined effect of GSH + NAA exhibited better rooting results than the individual effect of NAA alone, indicating that GSH and NAA act synergistically in the process of root regeneration at least in the MM106 apple rootstock. Similar findings and conclusions were reported by Imin *et al.* (2007) who found that both reduced (GSH) and oxidised (GSSG) form of glutathione markedly enhance the number of roots formed by callus derived from leaf explants of *Medicago truncatula* cultured on NAA-supplemented medium than on medium supplemented with NAA alone. In the current study with MM106 apple microshoots, GSH was applied only in combination with NAA since preliminary experiments (data not shown) showed that root formation was not stimulated due to GSH application alone in the absence of auxin. Our findings in MM106 apple rootstock are partly in line with those presented by Tyburski and Tretyn (2010) who demonstrated that supplementing the rooting medium with GSH (1-2.5 mM) increased the number of roots formed by tomato seedling cuttings grown on an auxin-free medium, however, the strongest stimulation of

root formation occurred when plants were simultaneously treated with auxin and GSH (Tyburski and Tretyn 2010). In MM106 apple rootstock, GSH when applied at 5, 50 or 1000 μM resulted in complete inhibition of rooting. Standardi and Romani (1990) reported inhibition of rooting in *Malus* due to GSH application at mM concentrations.

Shoot height of MM106 apple microcuttings was substantially enhanced due to GSH (2.5-500 μM) inclusion to the medium. The optimum GSH concentration of 10 μM resulted in an increase of shoot length by 2.81 cm in comparison to the control GSH-untreated MM106 apple explants. Our results are in agreement with those obtained by Nomura *et al.* (1998), who found that 100 μM GSH improved the development of isolated shoot tips of apple. Similarly, in soybean, 100 μM GSH increased shoot length both in the presence and absence of IAA shock alone (Auderset *et al.* 1996). Positive effects of GSH on shoot length were also reported for green onion (*Allium cepa* L., cv. Giza 6) plants (El-Awadi and Abd El Wahed 2012) and *Spilanthes calva* L. *vitro* culture (Shankar *et al.* 2012). In micropropagated MM106 apple shootlets, GSH (2.5-1000 μM) did not exert an inhibitory effect on vegetative growth. On the other hand, in gladiolus, shoot organogenesis frequency and shoot number per explant using leaf segment explants were increased with the addition of 500 μM GSH, however, higher concentrations were found to be inhibitory (Dutta Gupta and Datta 2003).

Callus growth of MM106 apple microcuttings regarding callus FW and callusing percentage was stimulated in a considerable degree by adding GSH to the medium. Similar findings were reported in apple *vitro* culture, where GSH promoted callus growth (Nomura *et al.* 1998), and also in *Pistacia vera* shoot tip culture (Tabiyeh *et al.* 2006) results indicated that GSH reduced the total phenolic compounds, promoting shoot growth. In the present study with MM106 apple rootstock, callus induction percentage was highest with 10 μM GSH. In yew (*Taxus baccata* L.), callogenesis percentage was significantly increased when 100 μM GSH were added to the culture medium (Ghafoori *et al.* 2012).

The mechanism by which thiol compounds might enhance rooting is unknown. For example, GSH reduces auxin effects by forming conjugates (Farago *et al.* 1994). However, there is no precedent for potentiation of an auxin response by thiols. Our findings may be of theoretical importance concerning a potential auxin x thiol interaction in plant growth and differentiation and of practical importance to artificial rooting of woody and herbaceous shoot microcuttings.

CONCLUSIONS

GSH participates in plant regeneration, being involved in mechanisms regulating cell divisions in newly formed meristems and participating in hormone metabolism and signalling. Diverse functions of this antioxidant open vast possibilities of using it for the improvement of tissue culture and plant regeneration methods. However, further studies are required to fully exploit the

properties of GSH for manipulating developmental processes in plant tissue culture..

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