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Efficient Adventitious Shoot Formation of Leaf Segments of *In Vitro* Propagated Shoots of the Apple Rootstock M.9/T337

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Summary

Several factors affecting *in vitro* regeneration of the difficult to regenerate apple rootstock M.9 type T337 have been examined by modifying the composition of tissue culture media, namely basal salt mixture as well as cytokinin (TDZ, BAP) and auxin (NAA) concentration and combination. MS medium (with Van der Salm modification) gave consistently better results than DKW medium. Highest regeneration rates (ca. 87 %) were obtained with 0.54 µM NAA/0.45 µM and 15.0 µM TDZ, respectively. However adventitious shoots were hyperhydric irrespective of the TDZ/NAA concentration. Replacing TDZ by 22.0 µM BAP resulted in a little lower regeneration rate (68 %) but here only 7 % of the explants were hyperhydric.

Key words. BAP – hyperhydricity – M.26 – regeneration – thidiazuron – tissue culture

Introduction

Regeneration of plants from adventitious shoots is a prerequisite for the utilisation of gene transfer technologies. Adventitious shoot production has been reported from leaf explants for several apple cultivars (James et al. 1988; Fasolo et al. 1989, De Bondt et al. 1994, 1996; Yao et al. 1995; Hanke et al. 1999; Aldwinckle et al. 2003; Szankowski et al. 2003; Wilson and James 2003; Seong et al. 2005) and rootstocks (James et al. 1988; Maheswaran et al. 1992; Famiani et al. 1994; Norelli et al. 1994; Pawlicki and Welander 1994; Holefors et al. 1998, 2000; Zhu et al. 2001; Höhnle and Weber 2007). Usually leaf discs or leaf segments cut from *in vitro* propagated plant material were used as the basic material for regeneration. However, important differences in the regeneration rate existed due to different genotypes. The dwarfing rootstock M.9 is used in commercial apple production worldwide. M.9 was selected at East Malling from a number of French genotypes collectively known as 'Juane de Metz'. Over the past decades numerous 'sub-clones' of M.9 have been selected including the 'sub-clone' M.9/T337 which currently is the predominantly used M.9 type. These selected 'sub-clones' differ slightly in their individual characteristics like e.g. propagation quality and their effect upon scion vigour (Webster and Wertheim 2003). Although all M.9 'sub-clones' are "genetically similar", they show ontogenetical differences (Webster and Wertheim 2003). However in the literature the 'sub-clones' of M.9 are quite often not distinguished explicitly. There are several reports about regenerating M.9 without specifying the 'sub-clone' (James et al. 1988, Welander et al. 1989). However, only few groups specify the M.9 type used in their experiments (Zhu et al. 2001; Höhnle and Weber 2007). They reported immense differences in regeneration efficiency varying from approximate 8 % in M.9 (type not specified) (James et al. 1988) to 100 % in M.9/29 (Zhu 2005, pers. comm.). The phytohormone thidiazuron (TDZ) is a very potent cytokinin promoting growth in *in vitro* cultures of woody plant species (Huetteman and Preece 1993). The importance of TDZ in promoting shoot formation of apple has been reported (Theiler-Hedtrich and Theiler-Hedtrich 1990). TDZ has been used (in combination with other plant growth regulators) during the regeneration phase of different M.9 'sub-clones'. The TDZ concentration varied from 2.7 µM (regeneration of Jork 9; Caboni et al. 1996) to 15 µM (regeneration of M.9/29; Zhu et al. 2001). Recently we have shown that the regeneration capacity of M.9/T337 is favourably influenced by sorbitol (Höhnle and Weber 2007).

The objective of the present study was to investigate the effect of various phytohormones namely benzyl amino purine (BAP), thidiazuron (TDZ), α-naphthalene acetic acid (NAA) and their concentrations on adventitious shoot formation of the difficult to regenerate apple rootstock M.9/T337. These data were compared to the well regenerating apple rootstock M.26, which is a cross of M.9 and M.16.

Materials and Methods

In vitro cultures of the apple rootstock M.9/T337 were propagated on proliferation medium as described by Zhu et al. (2001) with the following adaptations: MS salts and vitamins with Van der Salm modification (Van der Salm et al. 1994), 3.0 µM thiamine, 3 % (w/v) sorbitol and 0.8 % (w/v) Daishin agar (Duchefa). Whereas M.26 shoot cultures were maintained on propagation medium based on Yepes and Aldwinckle (1994) medium with the following alterations: 3.0 µM thiamine and 0.8 % (w/v) Daishin

agar (Duchefa). Shoots were incubated at 22 °C with a 16/8 h day/night photoperiod and subcultured every 4 weeks.

Experiment I: The following factors which have a potential to improve regeneration capacity were tested: Cytokinin type (BAP, TDZ) and concentration, auxin (NAA) concentration as well as medium composition (basal salt mixture). Concurrently two different salt mixtures were used: (i) the MS basal salt mixture with Van der Salm modification (Van der Salm et al. 1994) (MS-VdS) and (ii) the DKW basal salt mixture (Driver and Kuniyuki 1984) (Table 1). The media were furthermore supplemented with MS vitamins (Murashige and Skoog 1962) and 220 mM sorbitol, 0.25 % (w/v) gelrite at pH 5.5. The 4 youngest unfolded leaves of 4 weeks old *in vitro* propagated shoot cultures were used. Petioles and leaf apices were removed and the middle parts of the leaves were cut into stripes. Leaf stripes were placed on regeneration medium with their adaxial side in contact with the medium. Regeneration was carried out in TC-Petri dishes (Ø 94 mm; Sarstedt) with 10 leaf stripes per Petri dish (containing approx. 25 ml regeneration medium). Explants were cultivated in the dark at 24 °C until shoots were visible. Newly formed shoots were transferred to shoot formation medium consisting of MS salts and vitamins with Van der Salm modification (Van der Salm et al. 1994), 220 mM sorbitol, 1.1 µM NAA, 20 µM TDZ, 0.35 % (w/v) Daishin agar (Duchefa) and 0.12 % (w/v) Gelrite (Duchefa). They were incubated at 22 °C with a photoperiod of 16/8 h day/night. Subcultivation was performed every 2–3 weeks. Shoots of the length >10 mm were transferred to propagation medium (see above). Each regeneration treatment included 40 leaf segments of M.9/T337 and 20 leaf segments of M.26 respectively, and was repeated twice. The number of leaf stripes forming shoots was scored after 5 and 8 weeks. Whereupon all explants were counted as regenerants, if they contained one or more regenerated shoots.

Experiment II: The revised protocol for the regeneration medium consisted of MS-VdS basal salts, vitamins, sugar, gelling agent as mentioned above, and 22.0 µM BAP/1.0 µM NAA. The implementation of this experiment was the same as described above, with the following changes: 50 leaf segments per treatment were used and each treatment was repeated once.

Results and Discussion

In a preliminary experiment we tested M.9/T337 on regeneration medium optimised for M.9/29 according to Zhu et al. (2001), containing 5.4 µM NAA/15.0 µM TDZ. However, less then 10 % of the explants exhibited at least one regenerated shoot (data not shown). Therefore in a first regeneration experiment the combination of plant growth factors NAA and TDZ in culture media with respect to regenerating M.9/T337 was tested (Table 1).

Best regeneration rates for M.9/T337, namely ca. 87 % were obtained with MS-VdS media containing 0.54 µM NAA/0.45 µM TDZ and 0.54 µM NAA/15.0 µM TDZ, respectively (Table 1). In comparison, the M.26 rootstock regenerated best at rates of 100 and 97 %, respectively on media containing 0.54 µM NAA/45.4 µM TDZ (DKW and MS-VdS basal salts) (Table 1). On media allowing highest regeneration (87 %) of M.9/T337 shoots, M.26 developed shoots at even higher rates of 90 and 95 %, respectively. Generally speaking the MS-VdS salt mixture is clearly superior to the DKW basal salt mixture regarding the regeneration efficiency of M.9/T337. This medium was even more beneficial for regenerating M.26 (Table 1). The DKW basal salts were chosen, because Wilson and James (2003) reported of a beneficial effect of the DKW mixture on the regeneration efficiency of the apple cultivar 'Queen Cox'. However, our results did not show any advantageous effect of the DKW salts on regeneration efficiency of M.9/T337. The main difference between the two salt mixtures was their total nitrogen content as well as the NO_3:NH_4 ratio. The observed phenomena could be genotype specific. Therefore all further experiments were carried out only with the MS-VdS basal salt mixture.

Plant growth regulators play an important role in inducing shoot regeneration from tissue cultures. TDZ has been reported to stimulate shoot proliferation in apple (Van Nieuwkerk et al. 1986) and other woody plant species (Huetteman and Preece 1993). For regeneration from leaf explants of different apple cultivars and rootstocks TDZ has been used at a range between 0.1 µM and 15.0 µM by several working groups (Van Nieuwkerk et al. 1986; Elobeidy and Korban 1988; Fasolo et al. 1989; Pawlicki and Welander 1994; De Bondt et al. 1996; Zhu et al. 2001; Wilson and James 2003). Whereupon the optimal TDZ concentration appeared to be dependent on the genotype. For example Zhu (2005, pers. comm.) reported of 100 % regeneration of M.9/29 using 5.4 µM NAA/ 15.0 µM TDZ whereas we obtained the best regeneration efficiency for M.9/T337 using 0.54 µM NAA/0.45 µM TDZ (Table 1). It has been shown that the auxin NAA has a beneficial effect on shoot induction on apple leaf explants (Fasolo et al. 1989, Korban et al. 1992). It was striking that best regeneration rates of M.9/T337 at levels

Table 1. Effect of different NAA/TDZ concentrations and basal salt mixtures on regeneration of adventitious shoots (%) from leaf segments of apple rootstocks M.9/T337 and M.26.

NAA	TDZ	M.9/T337		M.26	
(µM)	(µM)	MS-VdS	DKW	MS-VdS	DKW
		% Regeneration[1)]			
0.54	0.45	87.3 c	0.8 a	90.0 eg	60.4def
5.37	0.45	16.1 b	0.0 a	26.1 bd	25.9 cd
26.90	0.45	0.0 a	0.0 a	5.0 abc	2.4 ab
0.54	15.00	87.2 c	0.0 a	95.0 fgh	57.0 de
5.37	15.00	0.6 a	0.6 a	5.0 abc	20.0 bd
26.90	15.00	0.0 a	0.0 a	1.9 abc	0.0 a
0.54	45.40	45.5 b	0.6 a	97.0 gh	100.0 h
5.37	45.40	0.6 a	0.0 a	0.0 a	18.0 bd
26.90	45.40	0.0 a	0.0 a	0.0 a	1.9 ab

[1)]Percentage of explants with one or more regenerated shoots after 8 weeks of cultivation. Means with same letter (per genotype) are not significantly different. Percentage data were subjected to logit transformation before statistical analysis and then transformed back for presentation.

Table 2. Regeneration of adventitious shoots of leaf segments of apple rootstocks M.9/T337 and M.26 as well as hyperhydricity under the influence of 1.0 μM NAA and 22.0 μM BAP.

Rootstock	Regeneration rate[1] (%)	Mean no. of shoots[2] regenerated per leaf segment	Hyperhydricity rate[3] (%)	Mean no. of hyperhydric shoots[2] per leaf segment
M.9/T337	68.0 ± 1.2	0.9 ± 0.1	7.0 ± 0.6	0.1 ± 0.1
M.26	85.7 ± 0.4	3.6 ± 0.3	23.0 ± 3.4	0.8 ± 0.3

[1]Percentage of explants with one or more regenerated shoots after 8 weeks of cultivation.
[2]Shoots ≥ 5 mm
[3]Percentage of explants with one or more hyperhydric shoots after 8 weeks of cultivation.

of about 87 % were obtained with the lowest NAA concentration (0.54 μM) whereupon increasing TDZ concentrations resulted in lower rates (Table 1). Similar observations were made for M.26 (Table 1). Higher NAA concentrations obviously hamper regeneration regardless of the TDZ concentration. Fasolo et al. (1989) mentioned analogous results. They reported best regeneration of adventitious shoots from apple leaf explants with NAA concentrations of 1.1 μM and 5.4 μM.

Hyperhydricity is a common phenomenon in tissue culture (Paques 1991; Ziv 1991). Several factors are responsible for inducing hyperhydricity like the gelling agent type, its concentration, humidity in the culture vessel and others like the type and dose of cytokinin (Ziv 1991). It has been reported that TDZ may cause hyperhydricity especially in higher concentrations and during prolonged exposure (Lu 1993). For example Gribaudo and Fronda (1991) showed for grapevine that increasing TDZ concentrations promoted hyperhydricity. Most of our regenerated shoots of M.9/T337 and also of M.26 showed clear signs of hyperhydricity on regeneration medium after 6 to 10 weeks of cultivation (data not shown). Similar observations made Pawlicki and Welander (1994) while regenerating Jork 9 on medium containing 1.0 μM TDZ. Due to the fact that most of the regenerated shoots became hyperhydric, we changed the composition of cytokinin content in the regeneration medium in the second experiment. TDZ was replaced by BAP (Table 2). The best regeneration rate of M.9/T337 namely 68 % (Table 2) was in fact lower in comparison to the first experiment (Table 1), but it is noteworthy that only 7 % of the explants had at least one hyperhydric shoot. Further optimising steps of the regeneration medium should increase the regeneration rate. The positive control for this experiment, M.26 regenerated with a rate of 85.7 % (Table 2) not as good as in the first experiment (Table 1). Our observation agrees with findings of Welander (1988) who observed for M.26 on a somewhat different regeneration medium (N6 macro elements, 22.0 μM BAP, 1.0 μM NAA) a similar regeneration rate as we did namely of 84 %. But on a "number of shoots per explant basis" she got the fourfold amount of shoots. This could be due to differences in composition of the medium (salt mixture) or to different culturing conditions. The hyperhydricity rate of M.26 was indeed considerably lower as in the first experiment but with 23 % (Table 2) was still too high.

To the best of our knowledge this is the first report, which explicitly describes the regeneration of the *in vitro* culture recalcitrant rootstock M.9 type T337. We were able to raise the regeneration rate of M.9/T337 from primarily less than 10 to 68 % by using a regeneration medium consisting of MS salts with van der Salm modification and 1.0 μM NAA/22.0 μM BAP.

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