

In vitro shoot multiplication of apple: comparative response of three rootstocks to cytokinines and auxin

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Summary: Shoot multiplication responses of rootstocks cvs. M.26, MM.106 and JTE-H to different concentration of BA, BAR and IBA in eight various combinations were tested on MS-medium. The effect of hormones depended on genotype, type of cytokinin and interaction of cytokinin and auxin. Shoot multiplication was significantly enhanced with the use of BAR as cytokinin. High multiplication rate could be achieved in cvs. M.26, MM.106 and JTE-H: 7.7, 6.9 and 9.9 shoots per explant, respectively.

Abbreviations: 6-benzylaminopurine riboside - BAR, 6-benzylaminopurine - BA, indole-3-butyric acid - IBA

Introduction

Developments in high-density planting-system in apple orchards required a low-cost nursery stock. One most common approach is propagation of apple rootstocks and scions using tissue culture methods. Production of fruit tree rootstocks *in vitro* was the first major commercial application of micropropagation techniques to tree crops (Zimmerman, 1986). The rapid method of *in vitro* propagation of fruit trees was introduced by Jones (1976). Micropropagation of apple rootstocks opened up new areas of research and fruit tree propagation. This tissue culture procedure makes the rapid production of virus-free and uniform propagation material possible in large quantities. Although several methods have been described (Snir and Erez, 1980, James and Thurbon, 1981, Modgil *et al.*, 1999), apple micropropagation has been accomplished with different success because different cultivars respond in different way during *in vitro* proliferation and rooting (Zimmerman and Broome, 1981, Zimmerman and Fordham, 1985). Therefore procedures developed earlier for micropropagation of apple rootstocks could not be extrapolated with the same success to another genotypes. Another aspect of *in vitro* propagation of apple cultivars, i.e. the risk of rejuvenation has been discussed earlier (Bubán *et al.*, 1993).

The aim of this study was to solve the problem of phenolic browning at the initiation of the culture and to examine the effects of different cytokinins beside various

concentrations of indole-3-butyric acid on the rate of shoot multiplication and the length of developed shoots of three apple rootstocks.

Materials and methods

In the experiments the following three rootstocks were examined: M.26, MM.106 and JTE-H. Explants from field grown trees were collected and surface sterilized. First step of sterilization was the washing in tap water with Tween-20 for one hour. After washing shoot apices with a small piece of stem were sterilized in 70% ethanol for 3 minutes, then in 0.1% HgCl₂ for 5 minutes. After surface-sterilization shoot tip explants were washed three times in sterilized distilled water and inoculated on initiation medium based on half-strength MS-medium (Murashige and Skoog, 1962) supplemented with 0.5 mg l⁻¹ BAR + 0.1 mg l⁻¹ IBA + 0.5 mg l⁻¹ GA₃. To reduce the phenol oxidation, initiation medium was supplemented with antioxidants (0.15 g l⁻¹ citric acid and 0.1 g l⁻¹ ascorbic acid) and adsorbents (1 g l⁻¹ polyvinyl pyrrolidone and 2.5 g l⁻¹ activated charcoal and perlite). After one-week-culture explants were placed on solid medium with the same compounds of initiation medium.

Shoots were subcultured and multiplied at 4-week intervals on medium-1 (Table 1) to get a sufficient shoot number for multiplication experiments. The response of different rootstocks to different concentration of BA, BAR and IBA in eight various combinations were tested (Table 1).

Table 1 Concentration of growth regulators in the culture media used in the experiments

| Media | Concentrations of hormones (mg l ⁻¹) | | | |
|----------|--|-----|-----|-----------------|
| | BA | BAR | IBA | GA ₃ |
| medium-1 | 0.5 | – | 0.1 | 0.2 |
| medium-2 | 0.5 | – | 0.3 | 0.2 |
| medium-3 | 1.0 | – | 0.1 | 0.2 |
| medium-4 | 1.0 | – | 0.3 | 0.2 |
| medium-5 | – | 0.5 | 0.1 | 0.2 |
| medium-6 | – | 0.5 | 0.3 | 0.2 |
| medium-7 | – | 1.0 | 0.1 | 0.2 |
| medium-8 | – | 1.0 | 0.3 | 0.2 |

Shoot multiplication experiments were carried out on MS-medium (Murashige and Skoog, 1962) supplemented with 100 mg l⁻¹ myo-inositol, 0.7% agar-agar, 3% saccharose and different combinations of plant hormones (Table 1). All media were autoclaved for 20 min at 121 °C and 10⁵ Pa. The pH of the medium was adjusted to 5.8 before autoclaving. Shoots were cut and cuttings of 4 cm each were placed on the different medium for four weeks in order to develop new shoots. Experiments were carried out in Kilner-jars (400 ml, 75 mm inside diameter and 85 mm long) and four cuttings were placed horizontally on 40 ml of medium in each jar. Cultures were grown at 22±2 °C with 16 h photoperiod provided by warm-white lamps (Tungsram) at a PPF of 105 µmol s⁻¹ m⁻². Each treatment consisted of at least 20 replicates and experiments were repeated at least three times.

After four weeks of cultures the length of new shoots developed and the rate of shoot multiplication were determined by counting the number of new shoots produced per explant. The statistical analysis was made using analysis of variance followed by Tukey's test.

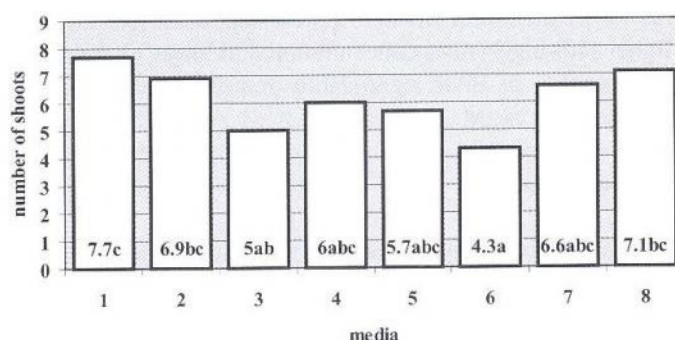
Results

Rate of shoot multiplication

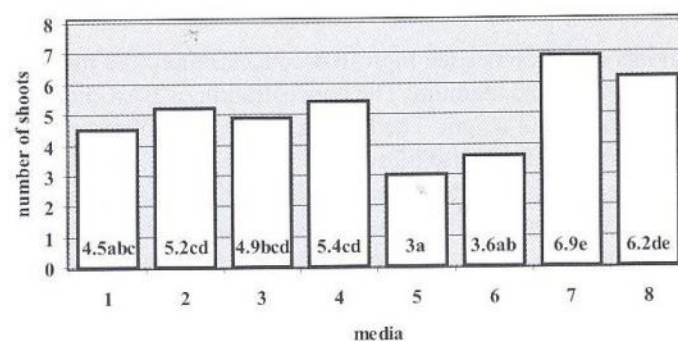
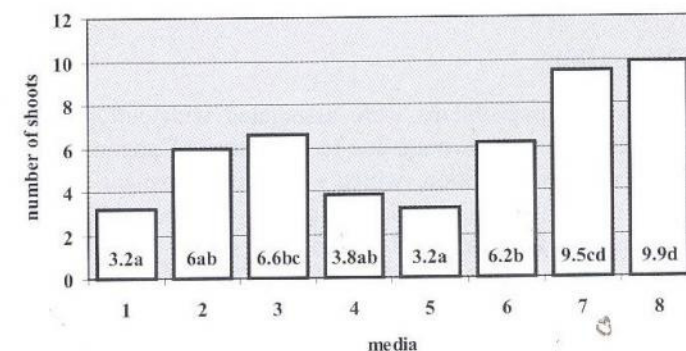
The shoot multiplication rate depended on genotype and on the hormone-content of the medium. The best multiplication rate was achieved with JTE-H (9.9 shoot per explant) on medium 8, with M.26 (7.7 shoots per explant) on medium 1, with MM.106 (6.9 shoot per explant) on medium 7 after 28 days (Figure 1–3).

IBA-content of the medium did not affect the number of shoots in cvs. M.26 and MM106 (Fig. 1 and 2). However, the number of shoots increased twofold if IBA-concentration was raised from 0.1 to 0.3 mg l⁻¹ combined with 0.5 mg l⁻¹ cytokinin regardless of type of cytokinin in the case of cv. JTE-H (Fig. 3).

The type and concentration of cytokinins affected the number of shoots in all rootstocks. Shoot multiplication was enhanced with use of BAR as cytokinin in cvs. JTE-H and MM.106 but only with higher concentrations. JTE-H and MM.106 rootstocks gave the best multiplication rate (9.9 and 6.9, respectively) on the medium containing 1 mg l⁻¹ BAR.

rootstock cv. M26**Figure 1** Effect of different combination and concentration of hormones on the rate of shoot multiplication in rootstock cv. M.26. The small letters mean the homogenous groups according to Tukey's test.

Lower concentration of BAR resulted in lower number of new shoots similarly to the effect of any concentrations of BA. Low multiplication rate was observed by use of BA in cvs. JTE-H (3.2 to 6.6) and MM.106 (4.5 to 5.4) (Fig. 2 and 3).

rootstock cv. MM106**Figure 2** Effect of different combination and concentration of hormones on the rate of shoot multiplication in rootstock cv. MM106. The small letters mean the homogenous groups according to Tukey's test.**rootstock cv. JTE-H****Figure 3** Effect of different combination and concentration of hormones on the rate of shoot multiplication in rootstock cv. JTE-H. The small letters mean the homogenous groups according to Tukey's test.

BA-content of medium was favourable only for M.26 concerning multiplication rate. The best multiplication rate was 7.7 by the use of BA in lower concentration (0.5 mg l⁻¹). Higher (1.0 mg l⁻¹) BA concentration and lower (0.5 mg l⁻¹) concentration of BAR significantly reduced the number of new shoots in cv. M.26 (Fig. 1).

Length of shoots

The length of new developed shoots depended both on genotype and medium (Table 2). In order to obtain longer

Table 2 Effect of different combination and concentration of growth regulators on the length of new developed shoots^a

| Length of shoots (mm) | Medium | | | | | | | |
|-----------------------|-------------|------------|------------|-------------|-------------|-------------|------------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| M.26 | 31.9 abc | 26.1 ab | 25.8 ab | 25.1 a | 31.2 abc | 37.6 c | 32.8 bc | 36.5 c |
| MM.106 | 36.7 a | 45.1 b | 44.9 b | 36.3 a | 39.3 ab | 44.9 b | 39.9 ab | 42.4 ab |
| JTE-H | 24.3 abc | 22 ab | 28.6 cd | 25.6 bcd | 31.5 d | 27.1 bcd | 22.1 ab | 19.6 a |

a: The small letters in rows mean the homogenous groups within the given genotype. Means within the rows followed by the same small letter are not significantly different at $p=0.05$.

shoots cv. M.26 needed high IBA-concentration (0.3 mg l⁻¹) and BAR in the medium. The concentration of BAR did not affect the shoot length. The longest shoots were observed on three media in the case of cv. MM.106 as follow. If BA was used as cytokinin, its effect on shoot length depended on IBA-concentration, that means beside low (0.5 mg l⁻¹) BA-concentration high IBA-concentration (0.3 mg l⁻¹) and beside high (1 mg l⁻¹) BA-concentration low (0.1 mg l⁻¹) IBA-concentration was necessary to obtain long shoots. When BAR was added into the medium, the most favourable combination of hormones was: 0.5 mg l⁻¹ BAR and 0.3 mg l⁻¹ IBA. The length of shoots was independent of IBA-concentration of the medium in cv. JTE-H. The concentration of cytokinin necessary for long shoots depended on type of cytokinin. High shoots were obtained on the media contained BA in 1 mg l⁻¹ or BAR in 0.5 mg l⁻¹.

Discussion

Two main problems were associated with culture initiation: explant browning due to oxidation of phenols and bacterial contamination, which also caused explant death. Rate of browning and contamination was dependent on season. Explants collected in winter or spring dyed mainly because of strong browning of buds. When the explants were collected during summer (shoots with well-expanded leaves) high grade of contamination could be observed but browning could be prevented well by using adsorbents and antioxidants. Although the seasonality of browning and contamination problems during establishment of *in vitro*

apple cultures were previously reported by others (Hutchinson, 1984, Webster and Jones, 1991, Modgil et al., 1999). Our observations were somewhat different in the time when the main problem was the contamination and browning, respectively. This difference in experiences may be related to the differences of climate where observations were made. Use of antioxidants (ascorbic acid, citric acid) and adsorbent (activated charcoal, polyvinyl pyrrolidone) during establishment was suggested by others (Zimmerman and Broome, 1980, Wang et al., 1994, Modgil et al., 1999) but browning could be hindered in our experiments only if they were applied in combination with perlite in liquid medium during first week of culture. Another factor in preventing phenol oxidation was the substitution of BA with BAR at the stage of culture initiation as reported earlier for *Prunus davidoper-sica* cv. Piroška (Jámbor-Benczúr et al., 1995) and *Ailanthus altissima* (Swingle) (Jámbor-Benczúr et al., 1997).

The best combination in the multiplication phase for high multiplication rate was different in the different genotypes after four-week culture. Although, the highest shoot number per explant was obtained on medium-1 containing 0.5 mg l⁻¹ BA and 0.1 mg l⁻¹ IBA in cv. M.26, shoot number obtained on medium-8 containing 1.0 mg l⁻¹ BAR and 0.3 mg l⁻¹ IBA was not significantly lower. However, for the best multiplication rate it was necessary the substitution of BA with BAR (1.0 mg l⁻¹) regarding the other two rootstocks. With the use of BAR the 5-fold multiplication rate per month described earlier (Snir and Erez, 1980) could be significantly exceed (6.9-fold multiplication rate on medium-7 containing 1.0 mg l⁻¹ BAR and 0.1 mg l⁻¹ IBA) in cv. MM.106. Similarly, 1.5-2.5-fold higher shoot number per month could be obtained with the use of BAR instead of BA in the case of cv. JTE-H. These data suggest that the effect of BAR can be favourable not only during culture initiation but it can increase the multiplication rate during proliferation phase. In JTE-H there was an other factor affecting multiplication rate: the auxin-concentration beside low (0.5 mg l⁻¹) concentration of cytokinin contrast with the two another rootstocks examined in our experiments and James and Thurbon's (1981) observations with the rootstock M.9.

The length of shoots was affected by interaction of cytokinin and auxin and in two of three rootstocks examined, the presence of BAR combined with IBA of higher concentration (0.3 mg l⁻¹) was favourable for obtaining longer shoots. In JTE-H the length of shoots was independent of IBA-concentration of the medium and the effect of cytokinin-type was concentration-dependent.

These data suggest that the effect of hormones depend on genotype and interaction of cytokinin and auxin, furthermore, a highly efficient method for multiplication can be established by the use of 6-benzylaminopurine riboside (BAR).

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References

- Bubán T., Balla I., Csubák E. & Tőkés G. (1993): Some doubts about using micropropagated trees in apple production. *Acta Horticult.* 349: 277-281.
- Hutchinson, J. F. (1984): Factors affecting shoot proliferation and root initiation in organ cultures of apple Northern Spy. *Scientia Hort.* 22: 347-358.
- James, D. J. & Thurbon, I. J. (1981): Shoot and root initiation *in vitro* in the apple rootstock M.9 and the promotive effects of phloroglucinol. *J. Hort. Sci.* 56(1): 15-20.
- Jámbor-Benczúr E., Tilly-Mándy A. & Szafián Zs. (1995): Effect of growth regulators on *in vitro* propagation of *Prunus X davidopersica* cv. Piroška. *Med. Fac. Landbouww. Univ. Gent.* 60/4a: 1665-1668.
- Jámbor-Benczúr E., Neményi A., Szendrák E. & Szafián Zs. (1997): *In vitro* propagation of *Ailanthus altissima* (Swingle) 'Purple Dragon'. *Horticult. Science* 29(1): 22-25.
- Jones, O. P. (1976): Effect of phloridzin and phloroglucinol on apple shoots. *Nature (London)* 262:392-393.
- Modgil, M., Sharma, D. R. & Bhardwaj, S. V. (1999): Micropropagation of apple cv. Tydeman Early Worcester. *Scientia Horticult.* 81: 179-188.
- Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Snir, I. & Erez, A. (1980): *In vitro* propagation of Malling Merton Apple rootstocks. *HortScience* 15(5): 597-598.
- Wang, Q. C., Tang, H. R., Quan, Q. & Zhou, G. R. (1994): Phenol induced browning and establishment of shoot tip explants of Fuji apple and Junhua pear cultured *in vitro*. *J. Hort. Sci.* 69: 833-839.
- Webster, C. A. & Jones, O. P. (1991): Micropropagation of some cold hardy dwarfing rootstocks for apple. *J. Hort. Sci.* 66: 1-6.
- Zimmerman, R. H. & Broome, O. C. (1981): Phloroglucinol and *in vitro* rooting of apple cultivar cuttings. *J. Am. Soc. Hort. Sci.* 106: 648-652.
- Zimmerman, R. H. & Fordham, I. (1985): Simplified method for rooting apple cultivars *in vitro*. *J. Am. Soc. Hort. Sci.* 110: 34-38.
- Zimmerman, R. H. (1986): Regeneration in woody ornamentals and fruit trees. In: Vasil I. K.: *Cell Culture and Somatic Cell Genetics of Plants*. Academic Press, Inc. Orlando, Florida. Vol. 3.: 243-258.