Plant Cell, Tissue and Organ Culture **27**: 155–160, 1991.

A new, efficient method using 8-hydroxy-quinolinol-sulfate for the initiation and establishment of tissue cultures of apple from adult material

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Received 21 December 1989; accepted in revised form 15 June 1991

Key words: browning, 8-hydroxy-quinolinol-sulfate, infections, in vitro-establishment, *Malus domestica*, micropropagation

Abstract

Applying the new method for culture initiation 16 different cultivars of *Malus domestica* could be established in vitro from shoot tips of adult orchard trees. Actively growing shoot tips were cleaned and surface disinfested, dissected to 2–3 mm and placed on a modified MS-medium with 4.4 μM BA. Explants were covered for 24 h with 200 μl of a 0.1% solution of 8-hydroxy-quinolinol-sulfate (8-HQS) and transferred to a medium containing both auxin and cytokinin after 2 weeks. The application of 8-HQS induced a strong reduction of the infection rate and inhibited the browning of the explants and the media. After 7 days yields of 50–90% sterile explants could be obtained in comparison to 100% losses of untreated shoot tips. After 60 days variable rates of actively growing shoots could be observed, depending on the genotypes. The described method allows a successful establishment of fruit trees from adult orchard material on one hand by strongly reducing the browning, caused by the oxidation of polyphenolic compounds by polyphenoloxidases, and on the other hand 8-HQS can strongly increase the yield of explants without contamination, independently from the vegetation period and the phytosanitary state of the donor material.

Abbreviations: BA – 6-benzyladenine, IBA – indolebutyric acid, 8-HQS – 8-hydroxy-quinolinol-sulfate

Introduction

Despite recent advances in micropropagation techniques, the establishment of woody plants has continued to be a difficult step. Keeping the donor plants under glasshouse conditions and pretreating them with antimicrobial substances allows considerable improvement of their phytosanitary state and decreases the infection pressure (Debergh 1987; Duhem et al. 1988; Enjalric et al. 1988). However, this is not always possible, especially if the donor plant is an old, mature tree in the field. In such cases losses of up to 100% can occur during in vitro-establishment. The most frequent problems are infections, endogenous bacteria and browning of explants. Additional problems with *Malus domestica* arise from the hairy surface, which necessitates longer disinfestation periods.

During preparation polyphenolic compounds, which exist in apple and many *Rosaceae* in high amounts, are oxidized by polyphenoloxidases (PPO). These oxidized compounds induce a strong browning of the cut surfaces and media and lead to the subsequent death of the explant (Hu & Wang 1983; Lê 1985; Skirvin et al. 1986), the most critical phase being the first 24 to 48 h in culture (R. Theiler, pers. comm.).

Many solutions have been proposed to solve these problems, e.g. the use of ascorbic acid, polyvinylpyrrolidone, activated charcoal, different disinfesting agents and culture conditions or antibiotics (Duhem et al. 1988; George & Sherrington 1984; Lê 1985; Poulsen 1988; Scortichini & Chiarotti 1988; Staritsky et al. 1984; Walkey 1972; Zimmerman & Broome 1981; Zimmerman 1984). Until now no generally applicable solution has been found. In this paper we describe a new method that allows a faster and safer initiation and establishment of fruit tree tissue cultures from adult material. During our attempt to establish tissue cultures of old local apple cultivars we encountered major problems at the initiation step, because some donor plants were very old trees. Many of these cultivars are not cultivated in commercial orchards, but are of great interest for an extensive plantation system, which in some parts of Austria still plays an important role and in some other parts is regaining importance. The fruits are used not only as fresh fruits for the market, but also for cooking and cider production. Tissue culture initiation of this material is posing major problems, because these trees have not been treated with fungicides for many years.

Materials and methods

Actively growing shoot tips from 16 different apple cultivars (Table 1) were collected from 20- to 40-year-old orchard trees from April (at the beginning of the vegetation period) until July (in the middle of the vegetation period).

Culture media were based on Murashige & Skoog inorganic salts and vitamins (1962) supplemented with 100 mg l^{-1} myoinositol, 20 g l^{-1} sucrose and 0.8% purified agar for microbiology (Merck, no. 1614). Medium A – a medium frequently used for the establishment of apple cultures (Zimmerman 1984), additionally contained 4.4 μM BA, medium B 2.64 μM BA and 0.15 μM IBA and medium C 1.58 μM BA and 0.15 μM IBA. All growth regulators were added to the media before autoclaving. Media were adjusted to a pH of 5.6–5.7 with 1N NaOH or 1N HCl and autoclaved for 20 min. Aliquots of 2.5 ml of media A and B were dispensed into each well of 24-well microtiter plates (Nunc). Ten milliliters of medium C were dispensed into 25 × 150 mm glass tubes.

Figure 1 shows the applied working steps for the in vitro-establishment of pomes. Shoot tips were cleaned and surface disinfested for 20 to 30 min in 10% NaOCl. Twenty-four shoot tips 2–3 mm long were dissected from each cultivar and placed on medium A. Control explants were kept in the same original wells for the following 10 days without any treatment.

Preliminary experiments with different concentrations of 8-HQS showed that a 0.1% solution of 8-HQS gave the best results. Treated explants were covered for 24 h with 200 μl filter sterilized 0.1% solution of 8-HQS (Chinosol, Riedel de Haen) and then transferred onto fresh culture medium of the same composition. Cultures were grown at 24°C ±2°C with a 16-h photoperiod provided by cool white fluorescent tubes with an irradiance of 100 μmol m^{-2} s^{-1}. After 10 to 14 days all explants were transferred to culture medium B, containing both cytokinin and auxin. The following passages were carried out at 3–4 week intervals on medium C.

The sterility and the browning of the explants after 7 days, their development after 20 days and the number of actively growing shoots after 60 days were recorded. Experiments were repeated several times from April until July at approxi-

Table 1. Apple cultivars used in this study with abbreviations used in Fig. 2.

BA – Baumanns Renette	BE – Berner Rosenapfel
CH – Champagnerrenette	GB – Grosser Bohnapfel
GE – Gelber Bellefleur	GO – Goldparmäne
JT – Jonathan	LA – Lavantaler Bananenapfel
LP – London Pepping	OB – Oldenburger
RB – Roter (Red) Boskoop	RK – Rheinischer Krummstiel
TH – Thurgauer Weinapfel	WB – Welschbrunner
WK – Weisser Winterkalvill	WO – Wondernot

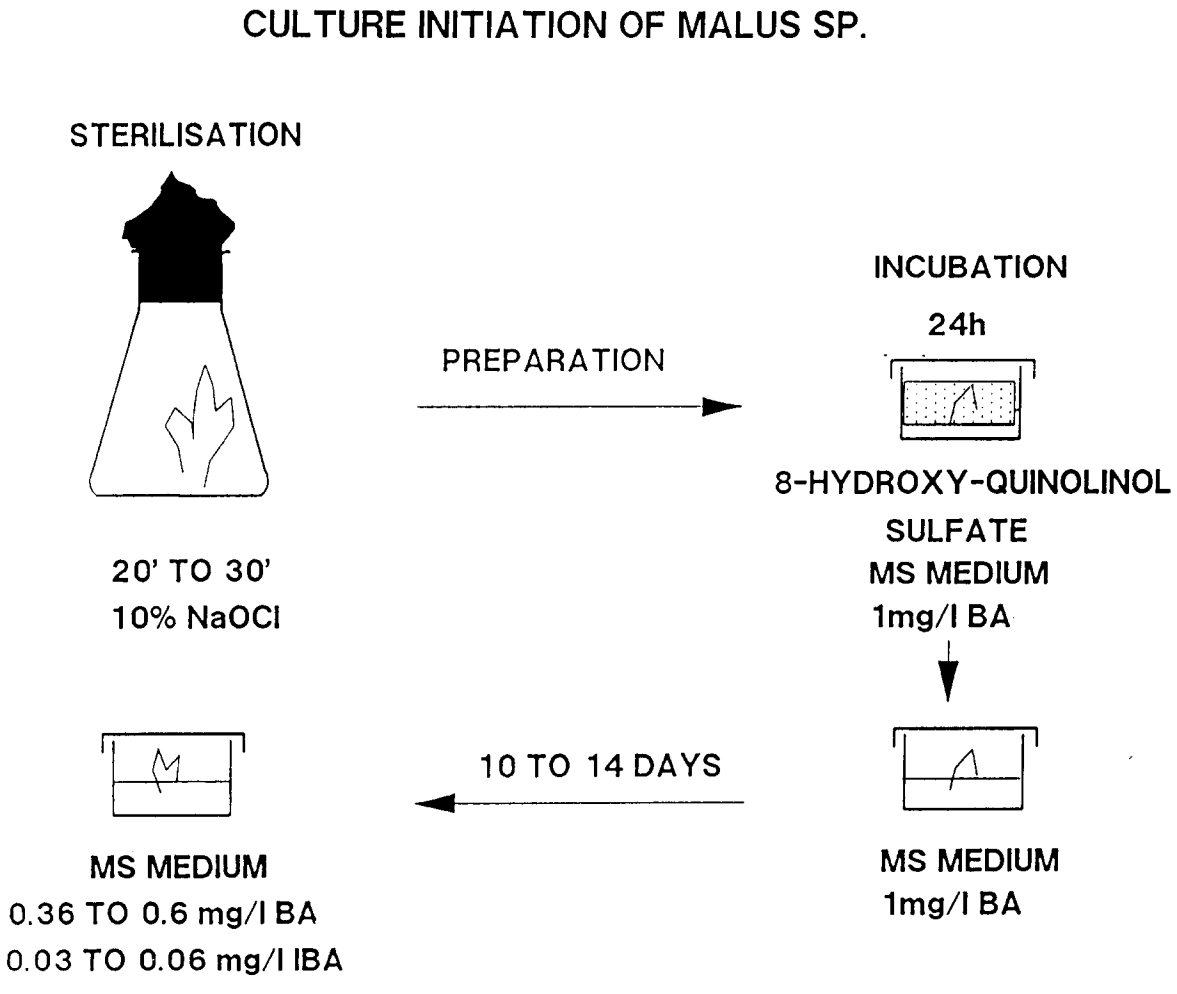

Fig. 1. Method for initiating cultures of mature apple trees using 8-hydroxy-quinolinol-sulfate.

mately monthly intervals in two consecutive years.

Results

In the experiments at the beginning of the vegetation period, when a low infection pressure is to be expected, a high rate of explants without contamination could be obtained from the different cultivars, when this method was applied. Figure 2A shows the results of the experiments during early months in the year (April). A range of 50 to 92% of uncontaminated explants were observed after 7 days (light bars, Fig. 2A). This was quite a progress, since in previous years, using the different methods cited above, we tended to lose most of our new cultures due to infections and browning already during the first week.

Even if the infection pressure was considerably higher in July, a comparable rate of successful establishment could be obtained (Fig. 2B). With the cultivar Jonathan using this method with 8-HQS, 92% of the explants established in April were uncontaminated after 7 days as were 50% of those established in July.

Treated shoot tips showed good development after 3 weeks in culture on medium B, where they developed 4 to 6 new leaves and elongated considerably (Fig. 3). The subsequent passage on medium C revealed quite strong differences

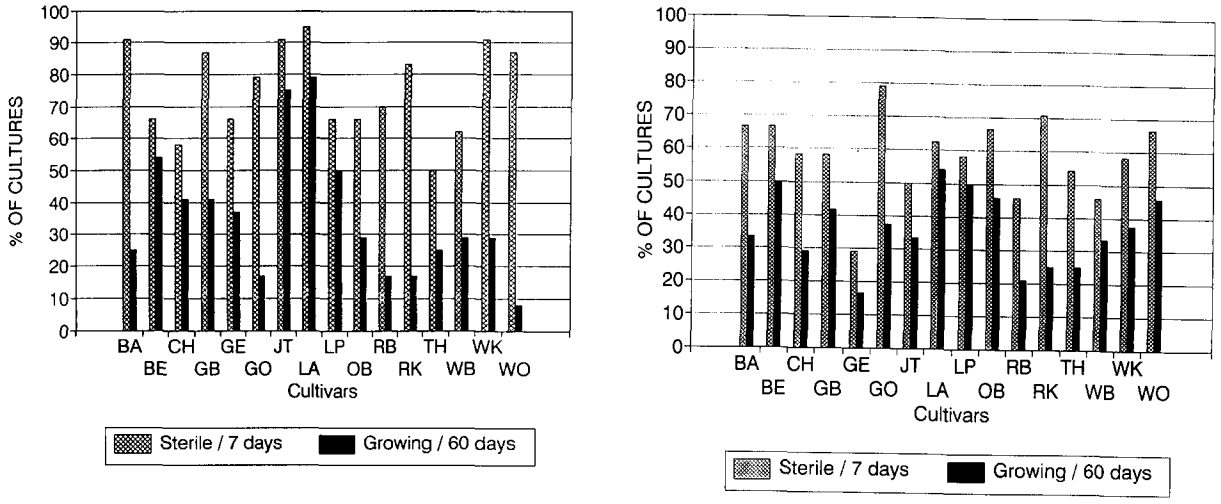

Fig. 2. Effect of establishment procedure on initiation of cultures from 16 apple cultivars from shoot tips collected in April (*A*) and July (*B*). See Table 1 for identification of cultivars.

in the requirements of the 16 cultivars (data not shown).

After 60 days, again varying among the cultivars, a range of 10–80% actively growing cultures was obtained (dark bars, Fig. 2). However, even starting from only 24 original explants for every cultivar it was possible, after 60 days, to have enough material from each cultivar to start experiments for the optimization of the multiplication medium when this method was used.

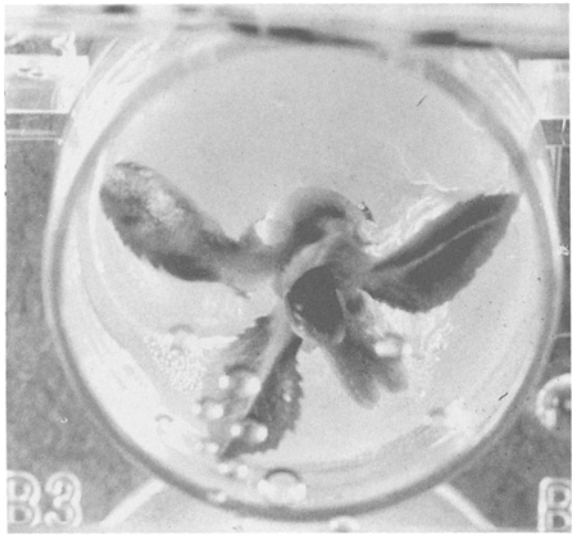

Fig. 3. In vitro shoot culture of cv. Großer Bohnapfel on medium B, 20 days after establishment.

Discussion

The unsatisfactory yield of sterile, actively growing shoots of fruit trees in-vitro using conventional methods of surface sterilisation has been documented by several workers (Enjalric et al. 1988; Hennerty et al. 1988; James & Thurbon 1978; Laimer et al. 1988; Webster & Jones 1989). Protocols for surface disinfestation that consistently yield a high number of uncontaminated explants would be highly desirable (Hennerty et al. 1988). It therefore appeared advantageous to use a substance that not only allowed a longer disinfestation period but also effectively inhibited the browning process without damaging the tissue or impairing later shoot development. We chose 8-HQS, an old antimycotic and bactericidal substance, which apart from its use in human healthcare (Auterhoff et al. 1991) has been used for treatment of budwood of fruit trees (K. Duhan, pers. comm.). Recently it has been shown that 8-hydroxy-quinoline citrate produced similar positive effects at the establishment of tissue cultures of woody species (Read & Yang 1990).

The application of 8-HQS during the establishment phase achieved a lower infection rate as well as resulting in a clear reduction of the browning caused by the PPO. 8-HQS seems to inhibit the polymerisation of phenols to the typical brown polymers, possibly by chelating essen-

tial metal ions (R. Ebermann, pers. comm.; Auterhoff et al. 1991; Cheng et al. 1982).

In most cases, losses, which appeared during the first 24 to 48 h after establishment of apple tissue cultures and were mainly due to browning of the explants, could result in a yield as low as 10% (Walkey 1972; Webster & Jones 1989). In contrast to this we have found that with the help of 8-HQS a yield of 50–92% could be achieved (Fig. 2). The differences in the rates of uncontaminated explants obtained after 7 days (light bars, Fig. 2) depend very strongly on the different phytosanitary stage of the donor plants in the field.

What we consider the great advantage of this new method is that even under a high infection pressure in the orchard at a later time in the vegetation period (July), comparable results could be obtained. The cultivar Jonathan is an outstanding example, showing an increase in uncontaminated explants from 5% to 50% using this method.

The various growing rates of the different cultivars after 60 days (dark bars, Fig. 2) depend on (*a*) the physiological stage of the donor plant (Collet & Lê 1987) and (*b*) the use of a uniform culture medium for the establishment of each of the 16 different genotypes.

It is our opinion that the latter point is the determining factor. We base this statement on the observations that the 16 cultivars showed different requirements in subsequently optimized multiplication media. For example the cultivar Baumanns Reinette tended to form single thick shoots on Medium C and showed a strong apical dominance, which corresponds to the natural habit of the cultivar. Only a change in the balance of growth regulators led finally to a satisfactory multiplication rate (data not shown). On the other hand also a possible infection with latent viruses could be the reason for the reduced vigour of the tissue and thus of the reduced number of actively growing explants. We deduce this from experiments with other genotypes, where starting material with a known viral infection was used, and which resulted in a significantly reduced establishment of cultures.

Finally the fact that such a long exposure to an antimicrobial substance does not harm the tissues suggests a possible solution of the problems encountered with endogenous bacteria. Experiments to verify this hypothesis are currently underway.

Acknowledgement

This work was supported by the Austrian Bundesministerium für Land- und Forstwirtschaft, Project Nr. 472.

References

Auterhoff H, Knabe J & Höltje H-D (1991) Lehrbuch der Pharmazeutischen Chemie, 12 Edition, Wiss. Verlagsges, Stuttgart

Cheng KL, Keihei Ueno & Toshiaki Imamura (1982) CRC Handbook of Organic Analytical Reagents. CRC Press, Boca Raton, Florida

Collet GF & Lê LC (1987) Micropropagation de porte-greffes de pommier et de poirier. Rev Suisse Vitic Arbor. Hort. 19: 253–259

Debergh PC (1987) Micropropagation of herbaceous plants. In: Alderson PG & Dullforce WM (Eds) Micropropagation in Horticulture – Practice and Commercial Problems (pp 27–36). University of Nottingham Trent Print Unit

Duhem K, le Mercier N & Boxus Ph (1988) Difficulties in the establishment of axenic in vitro cultures of field collected coffee and cacao germplasm. Acta Hort. 225: 67–75

Enjalric F, Carron MP & Lardet L (1988) Contamination of primary cultures in tropical areas: the case of *Hevea brasiliensis*. Acta Hort. 225: 57–65

George EF & Sherrington PD (1984) Plant Propagation by Tissue Culture. Exegetics Limited, Eversley

Hennerty MJ, Upton ME, Furlong PA, Harris DP, Eaton RA & James DJ (1988) Microbial contamination of in vitro cultures of apple rootstock M26. Acta Hort. 225: 129–137

Hu CY & Wang PJ (1983) Meristem, shoot tip and bud cultures. In: Evans DA, Sharp WR, Ammirato PV & Yamada Y (Eds) Handbook of Plant Cell Culture, Vol 1 (pp 177–227). Macmillan Publ, New York, London

James DJ & Thurbon JJ (1978) Culture in vitro of M9 apple. Rep. E. Malling Res. Stn. for 1977: 176–177

Laimer M, da Câmara Machado A, Hanzer V, Himmler G, Mattanovich D & Katinger H (1988) In vitro Vermehrung der alten Lokalsorte Graf Uhlhorns Augustkalvill (*Malus domestica*). Klbg Mitt. 38: 105–107

Lê CL (1985) Multiplication clonale in vitro du pommier (*Malus domestica* Borkh, var. Gravenstein) Rev Suisse Vitic Arbor Hort. 17: 311–315

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Poulsen GB (1988) Elimination of contaminating micro-

organisms from meristem culture of apple rootstock M26. Acta Hort. 225: 193–197

Read PE & Yang G (1990) New methods to obtain clean explants of woody species. In: Abstracts VIIth Intl. Congress on Plant Tissue and Cell Culture, Amsterdam, June 24–29, 1990: 128

Scortichini M & Chiarotti A (1988) In vitro culture of *Prunus persica* var. Laevis Gray: Detection of bacterial contaminants and possibility of decontamination by means of antibiotics. Acta Hort. 225: 109–118

Skirvin RM, Kouider M, Joung H & Korban SS (1986) The tissue culture of apple. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry, Vol 1 (pp 183–198). Springer Verlag, Berlin

Staritsky G, Hovers IH & Zandivoort EA (1984) Rifampicin, an effective antibiotic against bacteria in cultures in vitro. IAPTC Newsletter 43: 1

Walkey DGA (1972) Production of apple plantlets from axillary bud meristems. Can. J. Plant. Sci. 52: 1085–1087

Webster CA & Jones OP (1989) Micropropagation of the apple rootstock M9: effect of sustained subculture on apparent rejuvenation in vitro. J. Hort Sci. 64: 421–428

Zimmerman RH & Broome OC (1981) Phloroglucinol and in vitro rooting of apple cultivar cuttings. J. Amer. Soc. Hort. Sci. 106: 648–652

Zimmerman RH (1984) Apple. In: Sharp WR, Evans DA, Ammirato PV & Yamada Y (Eds) Handbook of Plant Cell Culture, Vol 2 (pp 369–395). Macmillan Publ, New York, London