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Optimization of culture media for *in vitro* rooting of *Malus domestica* Borkh. cv. Compact Spartan

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Abstract

Shoots of *Malus domestica* Borkh. cv. Compact Spartan raised *in vitro* do not root in a single auxin medium. Components of the rooting medium were tested not only for root initiation but also for root elongation. Root emergence and further growth were inhibited by a too prolonged auxin treatment, the presence of NH_4NO_3 and the lack of substrate aeration. Saccharose was essential to achieve highly reproducible root growth on agar but was not necessary on watered vermiculite. $Ca(NO_3)_2$ stimulated root initiation, emergence and growth and improved their viability.

Additional key words: agar, Ca^{2+}, NH_4^+, nutrients, saccharose.

Introduction

Rooting remains worrying for most of the *in vitro* propagators of fruit tree species especially the replication of high rooting rates and optimal root quality. The literature offers a wide range of chemical and physical treatments. The continuously increasing number of rootstocks and cultivars micropropagated on a large scale, should prove the efficiency and the control of such relatively empirical treatments. According to Sriskandarajah *et al.* (1990), however, it would seem that the physiology of adventitious root formation is not yet clearly understood.

Rooting success is often a function of the subculture number and of the culture conditions submitted to the shoots before rooting (Druart 1980, Sriskandarajah *et al.* 1982, Welander 1985, Webster and Jones 1989, Noiton *et al.* 1992). During the rooting stage, many interferences occurred with the culture medium components as well as with the environmental factors (Druart *et al.* 1982, Druart 1987). The complexity of these interferences (Zimmerman 1984a, Williams *et al.* 1985) makes the study of the rooting very hard to achieve. In our laboratory, *Malus* cv. Compact Spartan, although cultivated *in vitro* in most favourable conditions for successful

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rooting of other *Malus*, *Prunus* or *Cydonia* species (Druart *et al.* 1982, Druart 1987 and 1992, Duron *et al.* 1989), was always unable to root. The study of this abnormal behaviour led us to develop an extremely simple approach to the factors affecting the successive stages of root induction, initiation and elongation.

Materials and methods

Malus domestica Borkh. cv. Compact Spartan, was provided by the Institute of Agriculture of Waterford (Ireland). It was established *in vitro* by meristem culture (0.1 - 0.5 mm size) and has been micropropagated since 1983 following the method and the culture conditions previously described for several fruit-tree species (Druart 1980, 1988).

Our rooting method is very similar to that of Zimmerman (Zimmerman and Fordham 1985) including an auxin treatment followed by the transfer of the shoots to achieve root elongation.

The auxin treatment covers root induction and initiation and starts in complete darkness. The first experiments deal with the optimal period of auxin treatment by testing periods of 3, 5, 7, 10, 15 and 30 d, all the shoots being submitted to 16 h photoperiod after the 5th day. Simultaneously, a mixture of indole-3-acetic acid (IAA) (0.1 mg dm^{-3}), indole 3 butyric acid (IBA) (1 mg dm^{-3}) and saccharose (20 g dm^{-3}) was compared with a complete "NK" rooting medium, liquid or gelified by 5 g dm^{-3} agar (*Pastagar B*). NK rooting medium consists of 1/2 Lepoivre macronutrients and full Lepoivre micronutrients, thiamine HCl (0.4 mg dm^{-3}), meso inositol (100 mg dm^{-3}), IBA (2 mg dm^{-3}), saccharose (20 g dm^{-3}), pH adjusted to 5.6, as previously described (Druart *et al.* 1982), completed by a vitamin and amino acid complex called "NK complex" (Druart 1987 and 1992). This complex contains riboflavin (1 mg dm^{-3}), pyridoxine (0.1 mg dm^{-3}), nicotinic acid (25 mg dm^{-3}), Ca panthothenate (1 mg dm^{-3}), ascorbic acid (1 mg dm^{-3}), D2 vitamin water soluble (5 cm^3 dm^{-3}), K5 vitamin (5 mg dm^{-3}) and L proline (100 mg dm^{-3}).

Root elongation covers root emergence and further growth. It takes place after transfer of the auxin treated shoots on watered (100 cm^3 distilled water per 250 cm^3 vermiculite) vermiculite (grade 3) or on the gelified basal medium (BM) which is "NK" medium without "NK complex" and IBA. The influence of agar, agar + saccharose (2 %), agar + saccharose (2 %) + 1/2 macronutrients solution and agar + saccharose (2 %) + 1/2 macronutrients and full micronutrients solutions is compared on the root elongation of shoots previously treated by "NK" liquid medium during 5, 7, and 10 d.

For the further experiments, shoots dipping takes place during 5 d in the dark at 23 ± 1 °C. The replacement of the macronutrient solution of Lepoivre by its individual components, respectively NH_4NO_3 (0.20 g dm^{-3}), KNO_3 (0.90 g dm^{-3}), KH_2PO_4 (0.14 g dm^{-3}), $CaNO_3 . 4\ H_2O$ (0.60 g dm^{-3}) and $MgSO_4 . 7\ H_2O$ (0.27 g dm^{-3}) is investigated in the auxin treatment and in the root elongation medium agar + saccharose (2 %). The requirement of saccharose for root formation is finally confirmed in agar, undiluted aqueous extract of agar and distilled water.

Aqueous extract is obtained after 0.035 MPa pressures applied for one night on cooked and autoclaved agar (0.5 %) + water.

The rooting rate [(number of rooted shoots × 100)/ total number of shoots], the average number of roots per plantlet (A.R.N.) and the average length of the rooting system (A.R.L.) were recorded after 35 d of culture. Rooting rate was observed from the $12^{th} \pm 1$ day of shoots culture. The results were provided from 3 different subcultures of 21 shoots.

Results

Previous experiments revealed that only 3.1 % from a total of 455 initially induced shoots of *Malus* cv. Compact Spartan rooted in the "NK" rooting medium. The unrooted shoots did not form any friable callus which commonly characterizes excessive auxin stimulus. Nevertheless, they developed a root system after transfer on watered vermiculite.

Table 1. Comparison of the basal gelified medium (BM) and the vermiculite (V) as root elongation media on the rooting rate, total number of rooted shoots/total shoots treated ratio (R.S/T.S.) and the average root number (A.R.N.) as a function of the auxin treatment and the period of its application. Means + S.E.

Treatment	Medium	Rooting rate [%] 3 d	5 d	7 d	10 d	15 d	30 d	Total rooting R.S./T.S.	[%]	A.R.N.
IBA+IAA+	BM	57.1	81.0	76.2	47.6	42.9	76.2	80/126	63.5	2.0±0.5
saccharose	V	86.7	100	95.2	100	100	100	117/120	97.5	5.0±0.3
NK-liquid	BM	52.4	76.2	71.4	61.9	52.4	90.5	85/126	67.5	2.8±0.7
	V	85.7	100	100	100	90.5	100	118/123	95.9	5.1±0.8
NK-gel	BM	52.4	42.9	33.3	23.8	38.1	52.4	51/126	40.5	2.5±0.3
	V	90.5	100	100	100	100	90.5	114/126	90.5	4.4±0.8

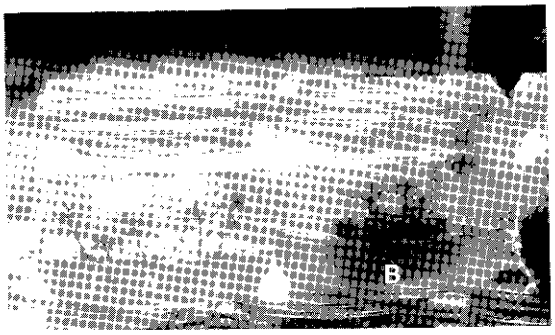

Fig. 1. Experiment to test the influence of the atmosphere on the root elongation: *A*) cotton join inserted under the cap of the jar allowing atmosphere exchanges; *B*) smaller jar containing the shoots to root in vermiculite introduced in the usual culture jar filled with basal medium.

The results relevant to the auxin treatment of the shoots (Table 1) show very reproducible rooting rates whatever the treatment conditions. 90 to 100 % shoots rooted on vermiculite, even after one month treatment. All the shoots were able to develop roots after a 5 d auxin treatment period. The rooting rate and average root number were lower when roots elongate on the gelified basal medium. Auxin treatment and root elongation, both carried out on gelified media, gave us the lowest rooting rate. Root emergence is therefore affected by *in vitro* growth conditions. Complementary trials consisting of the insertion of a cotton join under the cap of the jar, or of the introduction in the culture jar filled with the basal medium of a smaller jar containing the shoots to root on vermiculite proved that the rooting failure would not be ascribed to the closed atmosphere of the culture jar.

Among the different groups of medium components tested (Table 2), macronutrients were clearly involved in the inhibition process of root elongation.

Table 2. Rooting rate (mean ± S.E.) [%] as a function of the period of auxin treatment and components of the root elongation medium.

	Agar	Agar saccharose	Agar saccharose macro	Agar saccharose micro	Agar saccharose macro+micro	BM	Vermiculite
5 d	46.0±22.0	92.1± 7.9	73.0±15.6	100	65.1±16.6	63.5±15.1	85.3±12.0
7 d	48.8± 7.9	84.5±15.5	66.7±10.1	92.9±10.1	87.3± 4.2	63.5±20.3	85.7± 2.7
10 d	71.6±11.1	92.1± 7.9	61.9± 9.9	92.9±10.1	61.9± 8.3	63.5±11.1	89.0± 6.4
Total	55.1± 8.6	87.8± 7.1	66.7± 6.9	95.2± 3.0	71.4± 6.8	63.5± 8.0	86.8± 3.7

Saccharose is crucial for root growth on a gelified medium but unnecessary on vermiculite. The micronutrients could be stimulative. Among the different macronutrients in such elongation media, NH_4NO_3 inhibited partially or totally the root emergence and further growth (Table 3). Compared with the gelified medium containing only saccharose, ± 70 % of the plantlets cultivated in presence of NH_4NO_3 formed half as many roots and ± 30 % of the shoots remained unrooted. Root growth is also disturbed by KNO_3 which increased the frequency of growth arrests and reduced the final length of the rooting system (Table 3). Single effects of

Table 3. Rooting rate [%], average root number and average root length [cm] as a function of the presence of macronutrients during root elongation.

	Agar	Agar saccharose	Agar saccharose macro	Agar saccharose NH_4NO_3	Agar saccharose KNO_3	Agar saccharose $Ca(NO_3)_2$	Agar saccharose $MgSO_4$	Agar saccharose KH_2PO_4
R. rate	52.4±8.3	100	82.5±5.7	69.8±6.4	92.6±7.4	96.8±3.2	92.0±5.8	95.6±2.7
A.R.N.	2.55±0.58	5.14±0.79	2.83±0.19	2.55±0.46	4.79±0.84	4.79±0.84	4.11±0.95	4.49±0.76
A.R.L.	0.57±0.09	1.01±0.23	1.53±0.36	0.83±0.12	1.89±0.12	1.89±0.12	1.06±0.23	1.39±0.04

the other macronutrients could not be distinguished by the rooting rate and the average number of roots per plantlet. However, the longest roots are measured in the presence of KH_2PO_4 and $Ca(NO_3)_2$. NH_4NO_3 delayed the root formation (Fig. 2*B*). Roots emerged quickest in the presence of $Ca(NO_3)_2$ or $MgSO_4$. The latter ensured a final root development similar to agar + saccharose (2 %). In the presence of $Ca(NO_3)_2$, growth arrests did not occur and the root system showed a higher viability.

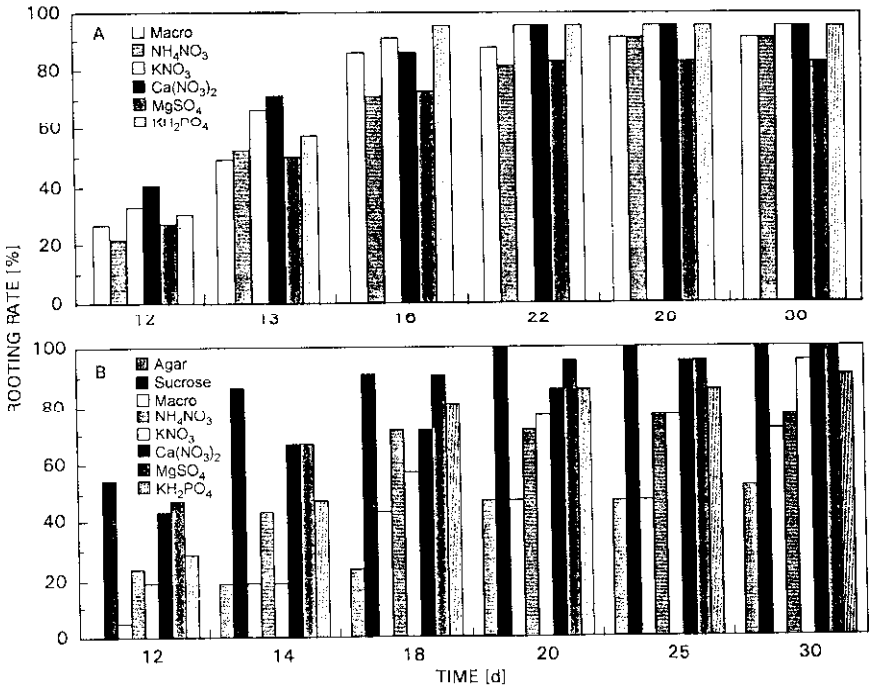

Fig. 2. Rooting rate [%] determined from the 12th day of shoot culture, as a function of the medium components during auxin treatment (*A*) or during root elongation (*B*). *A*: macronutrients tested in the 5-d auxin treatment of the shoots; roots elongating on agar + saccharose (2%). *B*: components of the root elongation medium tested after a 5 d of auxin treatment in "NK" liquid medium.

Separately tested during the auxin treatment, the macronutrients had no marked effect on the final rooting performances (Table 4). However, a delay in root emergence (Fig. 2*A*) was observed after auxin treatment in the presence of NH_4NO_3. Conversely, it was hastened by other macroelements mainly $Ca(NO_3)_2$ and KNO_3.

Saccharose is required for the roots growing on agar (Tables 2, 3 and 5), in aqueous agar extract or in distilled water (Table 5) but not on watered vermiculite. However, complementary trials showed that aqueous vermiculite extract required the addition of saccharose to ensure root formation (Druart, unpublished data). Vermiculite is, in fact, an aerated substrate in comparison with gel and unshaken water.

Table 4. Rooting rate [%], average root number and average root length [cm] as a function of the macronutrients present in auxin liquid medium.

	Macro	NH_4NO_3	KNO_3	$Ca(NO_3)_2$	$MgSO_4$	KH_2PO_4
R. rate	92.1	93.6±3.2	96.8±3.2	95.2	88.5±5.7	95.2±2.8
A.R.N.	5.08±0.31	4.78±0.17	5.72±1.10	4.28±0.81	4.69±1.58	5.37±0.71
A.R.L.	1.31±0.29	1.20±0.08	1.13±0.19	1.39±0.18	1.46±0.20	1.22±0.12

Table 5. Rooting rate [%], average root number and average root length [cm] as a function of the absence (*A*) or presence (*B*) of saccharose.

	Rooting rate		A.R.N.		A.R.L.	
	A	B	A	B	A	B
Agar	52.4	100	3.36±2.01	6.68±2.89	0.73	1.41
Agar extract	52.4	100	3.00±1.79	5.71±2.72	0.67	0.51
Distilled water	28.6	71.4	2.27±1.49	6.67±3.67	0.50	0.39

Discussion

Woody fruit species could be listed within 3 classes according to their required rooting treatment. The easy rooting species exhibit high quality rooting after a single auxin treatment or even without any treatment. A second group of species requires a complementary rhizogenous stimulus by a darkness treatment of the shoots at the begining of the rooting stage. The difficult rooting species require a supplementary treatment like the addition of vitamin D2 or L-proline (Druart 1987, Orlikowska 1988) or L-arginine (Orlikowska 1992) or potassium humate (Baraldi *et al.* 1991), *etc.*, to increase the auxin effect in the dark. The total auxin activity given to the shoots by cumulating all the physiological, physical and chemical factors have to be controlled to ensure optimal root induction and initiation. Too long treatment acts like an auxin excess which leads to rooting inhibition (James 1983, Zimmerman 1984b, Welander 1985) and even to disturbances of the shoots apical growth (Snir and Erez 1980). The auxin treatment must be appropriately interrupted by transplanting the induced shoots on a hormone free medium (James and Thurbon 1979, Snir and Erez 1980, Collet and Le 1985 and 1988, Vintherhalter and Neskovic 1992) possibly with the addition of some antioxidants (Standardi and Romani 1990) or by increasing the photooxydation of the auxin in the presence of riboflavin (Gorst *et al.* 1983, Druart *et al.* 1983, Drew *et al.* 1991).

Our preliminary trials led us to classify the apple cultivar Compact Spartan among the species rooting with difficulty *in vitro*. Indeed, our most efficient rooting conditions which combined IBA (2 mg dm^{-3}), 5 to 7 d of darkness and a specific organic complex NK treatment did not succeed in rooting the shoots. Total absence of friable calli at the stem section theoretically excluded any excessive auxin activity

(Favre 1977, Mitsuhashi-Kato *et al.* 1978, Collet and Le 1987, Welander and Snygg 1987) as a potential cause of root growth inhibition. On the contrary, such an observation would entail a stronger auxin treatment.

The successful rooting after transfer of the induced shoots to watered vermiculite demonstrated, on the contrary, that the auxin stimulus was sufficient and perfectly recorded by most of the shoots; only root emergence and growth were inhibited on the complete *in vitro* rooting medium. The hormonal compounds, the NH_4^+ ion and the gel were distinctly identified as inhibitors of the root elongation process of cv. Compact Spartan.

Three days of auxin treatment seemed sufficient for rooting almost all the shoots. Five days, which was the suitable culture time to achieve the root initiation of the apple cv. Jonagold (Druart *et al.* 1982), insured reproducible high rooting rates under our culture conditions. This period varies from one species to another and even from one shoot to another, all the shoots being physiologically different at onset of rooting. With the apple rootstock M9, only 12 to 48 h is the required period of shoots contact with IBA (James 1983).

Lowering the minerals to 1/2, 1/3 even 1/4 of the initial concentration generally leads to improved in *vitro* rooting of the woody fruit species (Quoirin *et al.* 1977, Simmonds 1983, Travers *et al.* 1985, Nemeth 1986). Our present work confirmed that NH_4NO_3 disturbed the rooting of cv. Compact Spartan as previously described Sriskandarajah *et al.* (1990) with the apple cvs. Gala and Jonagold. The effect of NH_4NO_3 is clearly an inhibition of root emergence and further growth which is characterized by a decrease in the average root number per plantlet, smaller roots and lower rooting rate. When added to the auxin medium, NH_4NO_3 could delay root emergence the longer the treatment is, but would not affect final rooting performances. Depending on the plant species, a high N content of the shoots is sometimes favourable (Tripathi 1971), sometimes detrimental (Roeber and Reuther 1982) to their rooting. Optimizing the C/N ratio in the culture media contributes to successful *in vitro* rooting of several species (Georges and Sherrington 1984). Elevated NH_4NO_3 concentrations allowed the spontaneous rooting of *Philodendron* to be suppressed, during multiplication (Sriskandarajah and Skirvin 1991).

Among the other mineral nutrients, Ca^{2+} played an important role during the entire rhizogenous process of *Malus* cv. Compact Spartan. Added to the rooting medium from the auxin treatment stage, Ca^{2+} stimulated root growth by quickening further root emergence from a higher number of root meristem tips and by maintaining optimal root viability during the elongation step. According to Blazich (1988), the role of Ca^{2+} is related to its fundamental involvement in the cell division and elongation process occurring during the root initiation phase. Mengel and Kirkby (1982) state that the growth of adventitious pea roots is impossible to maintain in the absence of Ca^{2+}; browning, growth decline and even root tips necrosis occured after discarding Ca^{2+} supply. The growth of Compact Spartan roots on gelified media free of Ca^{2+} often stops in the absence of minerals or in the presence of NH_4NO_3 or KNO_3, blushing occurs in the absence of minerals or in the presence of $MgSO_4$ and browning in the presence of NH_4NO_3, KNO_3, $MgSO_4$ or in the absence of any minerals. Variability of the rooting inhibition due to NH_4NO_3 was observed as a

function of the chosen agar (complementary unpublished data). This could be explained particularly by the Ca^{2+} release as an impurity which hardly differs from one agar brand to the other. The promotive effect of potassium as KCl on adventitious root formation was demonstrated in some herbaceous plants (Zhao *et al.* 1991). In our experiments, KNO_3 appeared favourable for Compact Spartan rooting when applied during the auxin treatment but it slowed down further root growth. Baraldi *et al.* (1991) pointed out the reducing effect of K-humate on root growth of the cv. Golden Delicious. $MgSO_4$ had no beneficial effect on the rooting of cv. Compact Spartan during auxin treatment but favoured root elongation. NH_4^+ inhibiting and Ca^{2+} stimulating effects were ultimately confirmed by repeating our rooting elongation experiment with Gresshof and Doy (1972) macronutrients where these minerals are added as $(NH_4)_2SO_4$ and $CaCl_2$, respectively (Gruselle, personal communication).

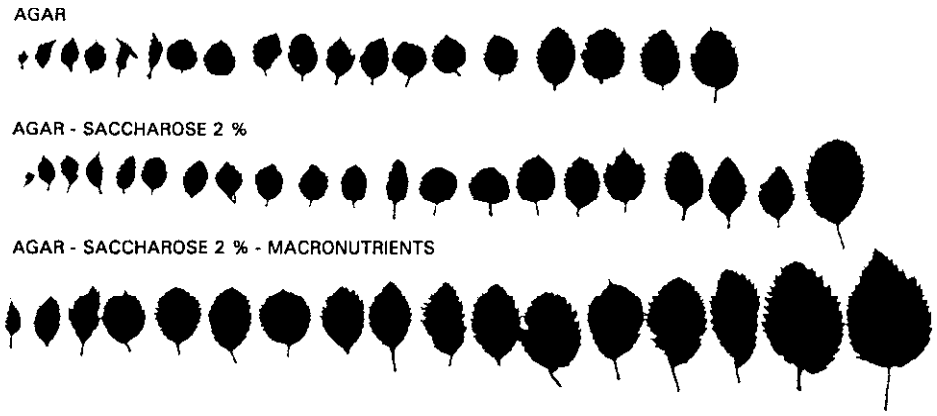

Fig. 3. First expanded leaf of plantlets rooted on agar, agar + saccharose (2 %) or agar + saccharose (2 %) + macronutrients.

Unlike cv. Antonovka 313 (Travers *et al.* 1985), cv. Compact Spartan did not need saccharose for rooting on watered vermiculite but did, however, on distilled water, agar and aqueous extracts of agar. The macronutrients only contributed to limb enlargement of neoformed leaves (Fig. 3) and to further growth of plantlets during their acclimatization. Previously Werner and Boe (1980) and Hutchinson (1984) also described the poor root growth of cv. Northern Spy in agar. Liquid medium even promoted the rooting of sweetgum (Lee *et al.* 1986). Unshaken solutions decreased the ability of rooting of the apple cv. Granny Smith (Sriskandarajah and Mullins 1982). Foam substrates aeration (Gebhart 1985) significantly improved root development of raspberries bushes. Recently, the highest rooting rates were obtained with walnut clones by adding vermiculite to the culture medium (Jay-Allemand *et al.* 1992), which contradicts Hutchinson's previous observations (1984). Our results confirmed that the addition of saccharose to unshaken solution and agar would offset the lack of root aeration. The better aeration is present in the vermiculite replacing agar.

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