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Micropropagation

Effects of phenols, gibberellic acid and carbohydrates on the rooting of the apple rootstock M9 Jork

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Summary — The rooting of the apple rootstock M9 Jork was increased by addition of phloroglucinol (162 mg/l) during rooting in the light in presence of 2 mg/l IBA. Other phenols (catechol, chlorogenic acid, *p*-coumaric acid and quercetin) did not show the same synergic effect. Phloroglucinol had no effect when added during the multiplication stage. GA_3 added during multiplication enhanced the rooting ability somewhat at a low concentration (1 mg/l), but was inhibitory at higher dosages. Sorbitol when used as carbon source during multiplication gave the best results in comparison with other sugars. Peroxidase activity was lower in the microcuttings rooted with the addition of phloroglucinol than in those grown in the presence of the other phenols or without phenols. Electrophoretic patterns of isoperoxidases did not show any particular changes due to the treatments.

***in vitro* rooting / carbon source / peroxidase**

Résumé — Effets du phenol, de l'acide gibbérellique et des glucides sur l'enracinement du pommier porte-greffe M9 Jork. *La capacité à l'enracinement du porte greffe M9 Jork du pommier a été augmentée par un traitement avec du phloroglucinol (162 mg/l) quand l'induction à l'enracinement des microboutures a été faite en présence de 2 mg/l de AIB et une photopériode de 16 h. D'autres composés phénoliques, catéchol, acide chlorogénique, acide p-coumarique et quercétine ne montrèrent pas les mêmes effets synergiques.*

Des résultats variables suivant la dose ont été obtenus avec l'utilisation de GA_3 alors que le sorbitol utilisé comme source de carbone donnait les meilleurs résultats.

L'activité péroxidasique était inférieure dans les microbutures enracinées avec l'addition de phloroglucinol à celle des boutures mises en présence d'autres phénols ou sans phénols. Les spectres isopéroxydasiques ne montrèrent aucun changement dû aux différents traitements.

enracinement* in vitro / *source de carbone / peroxydase

INTRODUCTION

The rooting ability of apple cuttings is highly genotype-dependent and the response to rooting treatments is variable (Zimmerman and Broome, 1981). A large number of investigations have been made on the effect of exogenous phenols added to the rooting medium, but with contradictory results: the widely used phloroglucinol is successful in some cases (James and Thurbon, 1979, 1981; Zimmerman, 1984), but has no effect or is even inhibitory in other instances (Zimmerman and Broome, 1981; Zimmerman and Fordham, 1985). The rooting process is regulated, among other substances, by auxin. Enzymes such as peroxidases, IAA-oxidase and polyphenoloxidases are implicated in IAA catabolism and in the oxidation of phenols which act as rooting cofactors or inhibitors. The effect of phenols and other cofactors depends on the interaction between these molecules and the enzymes implicated in the IAA-metabolism during the rooting process. It seems that monophenols and *meta*-diphenols activate IAA-oxidase, and that *para*-diphenols, *ortho*-diphenols and polyphenols inhibit it (Reinecke and Bandurski, 1990).

The aim of this study was to investigate the effect of various treatments during the multiplica-

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tion and rooting phase. We also determined peroxidase activity in order to examine the biochemical relationship between the treatments and their effect on rooting.

MATERIALS AND METHODS

Microshoots of the apple rootstock M9 Jork multiplied on MS medium with sucrose 10 g/l, sorbitol 20 g/l, IBA 0.1 mg/l, BAP 0.5 mg/l, agar 6.5 mg/l, pH 5.6 and rooted on a medium as indicated in table I, were used. Temperature was 24 °C, light intensity was 25 μmol m^{-2} s^{-1}, with a photoperiod of 16 h. The following treatments, on 60 shoots divided into 3 replicates, were applied:

– darkness during the first 7 d of rooting;

– phloroglucinol (PGL, polyphenol) 162 mg/l during the rooting phase, with or without 2 mg/l IBA;

– phenols; catechol (*o*-diphenol), quercetin (*o*-diphenol), chlorogenic acid (CGA, *o*-diphenol) and *p*-coumaric acid (monophenol) 1 mM during the rooting phase;

– PGL 162 mg/l in the multiplication medium, followed by rooting with or without PGL at 162 or 1 620 mg/l;

– multiplication with sucrose 10 g/l replacing sorbitol with equimolar quantities of fructose, glucose, maltose or galactose, followed by rooting with PGL 162 mg/l;

– multiplication with GA_3 et 1, 2 or 4 mg/l added to the medium before autoclaving, followed by rooting with PGL 162 mg/l.

Peroxidase activity was analyzed as follows. Samples of 200 mg of the basal part of the stems of the microcuttings were homogenized in 1.5 ml 0.06 M phosphate buffer, pH 6.1 with 150 mg polyvinyl–polypyrrolidone and centrifuged at 10 000 *g* for 10 min to extract the enzymes. Peroxidase activity was detected using guaiacol and H_2O_2 as substrates in phosphate buffer pH 6.1. The increase in absorbance at 470 nm was measured (Angelini *et al,* 1990). Electrophoresis was performed using starch gel and lithium–borate buffer, pH 8.3 (Quarta and Arnone, 1987); isoelectric focusing was also applied using Servalyte precotes, pH 3–10 (Serva). Peroxidases were stained by amino-ethyl-carbazole or by benzidine with H_2O_2 in acetate buffer pH 4.5 (Vallejos, 1983).

Table I. M9 Jork rooting medium composition.

Macro	Lepoivre	1/2	
Micro	Lepoivre	Full	
FeNaEDTA		40	mg/l
Nicotinic acid		0.5	"
Pyridoxine		0.5	"
Thiamine HCl		1	"
Inositol		100	"
Riboflavin		1	"
Proline		100	"
Sucrose		30	g/l
Agar		6	"
IBA		2	mg/l
pH 5.6			

Lepoivre = Quoirin *et al,* 1977.

RESULTS

Treatments during the rooting phase

The rooting percentage of M9 Jork was 30% with 2 mg/l IBA in the light. Culture for 7 d in the dark did not increase rooting, and callus production was higher (table II, fig 1).

Good results were obtained with the addition of 162 mg/l PGL during the rooting phase: it showed a synergic effect with IBA and increased rooting percentage both in the dark and in the light (fig 2). A higher dosage (1 620 mg/l) was inhibitory. Other phenols, such as catechol and CGA seemed to stimulate rooting somewhat when added to rooting media without IBA (table II). Their use together with the auxin had an inhibitory effect on the outgrowth of the roots (table II, fig 3). *p*-Coumaric acid was tested only with IBA in the light; it gave results similar to that of the other phenols (rooting 30%, 2.0 ± 0.3 roots per shoot), but the roots were more evident, although still very short.

Treatments during the multiplication phase

The use of PGL as pretreatment during multiplication did not improve the results: after 1 or 2 subcultures, rooting was the same as the control. The effect of PGL during the rooting phase was more evident without pretreatment (data not shown).

The multiplication rate decreased somewhat when sorbitol was replaced by other carbohydrates (table III), and no substantial differences were induced by gibberellic acid (fig 4). The dosage of 1 mg/l GA_3 during multiplication seemed to promote rooting on medium containing PGL at 162 mg/l, increasing the percentage from 67.4 to 75%. GA_3 at dosages of 2 and 4 mg/l inhibited rooting (rooting percentages ≈ 50–55%) (fig 4).

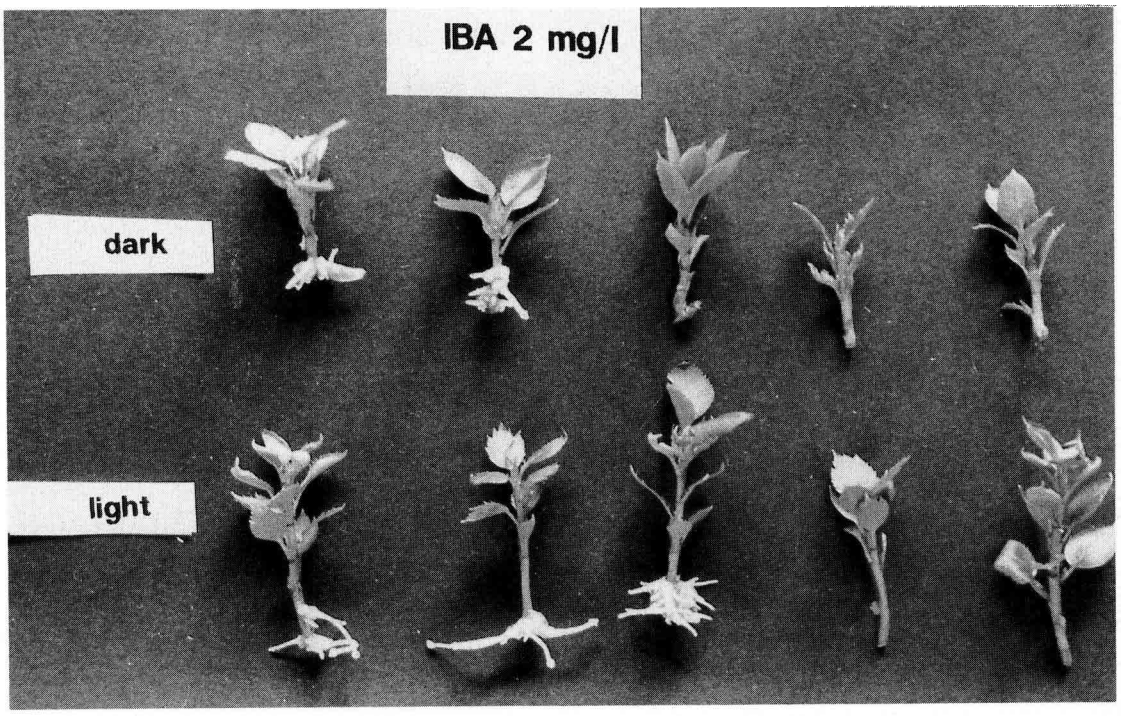

Fig 1. Rooting of M9 Jork, effect of dark treatment during the 1st wk of rooting.

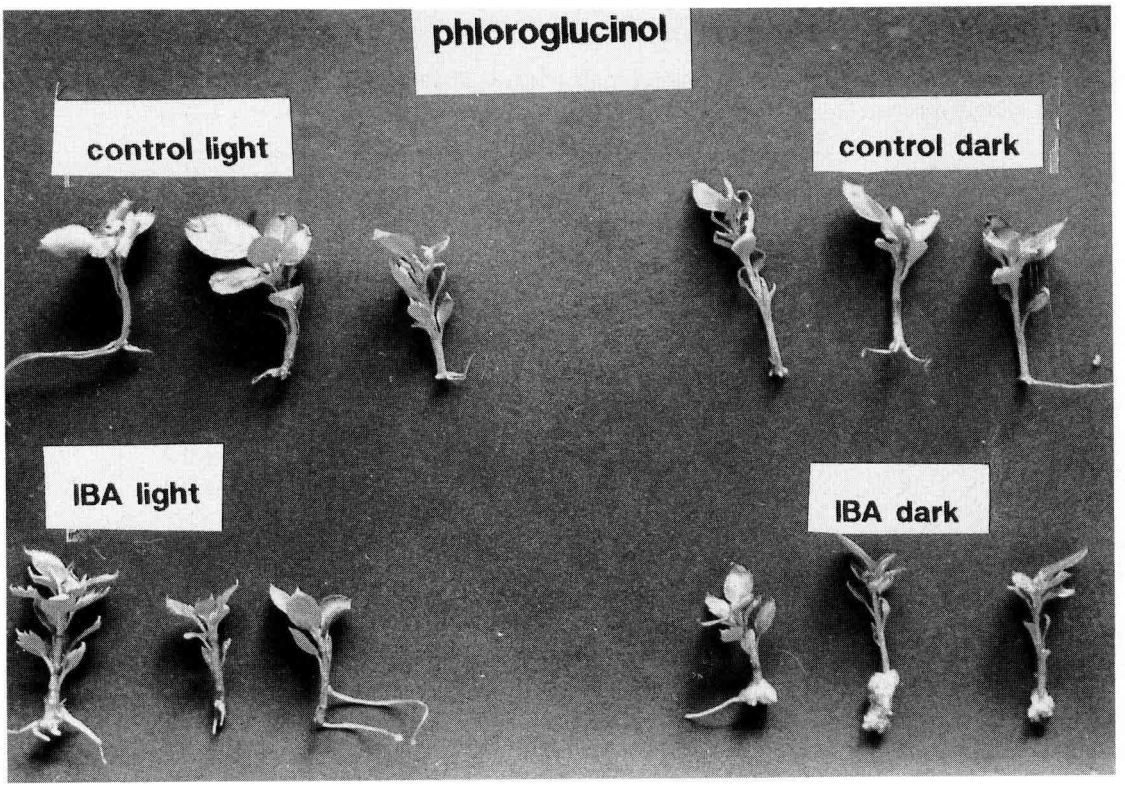

Fig 2. Rooting of M9 Jork with PGL 1 mM, with or without IBA 2 mg/l, in the light or with 7 d in the dark.

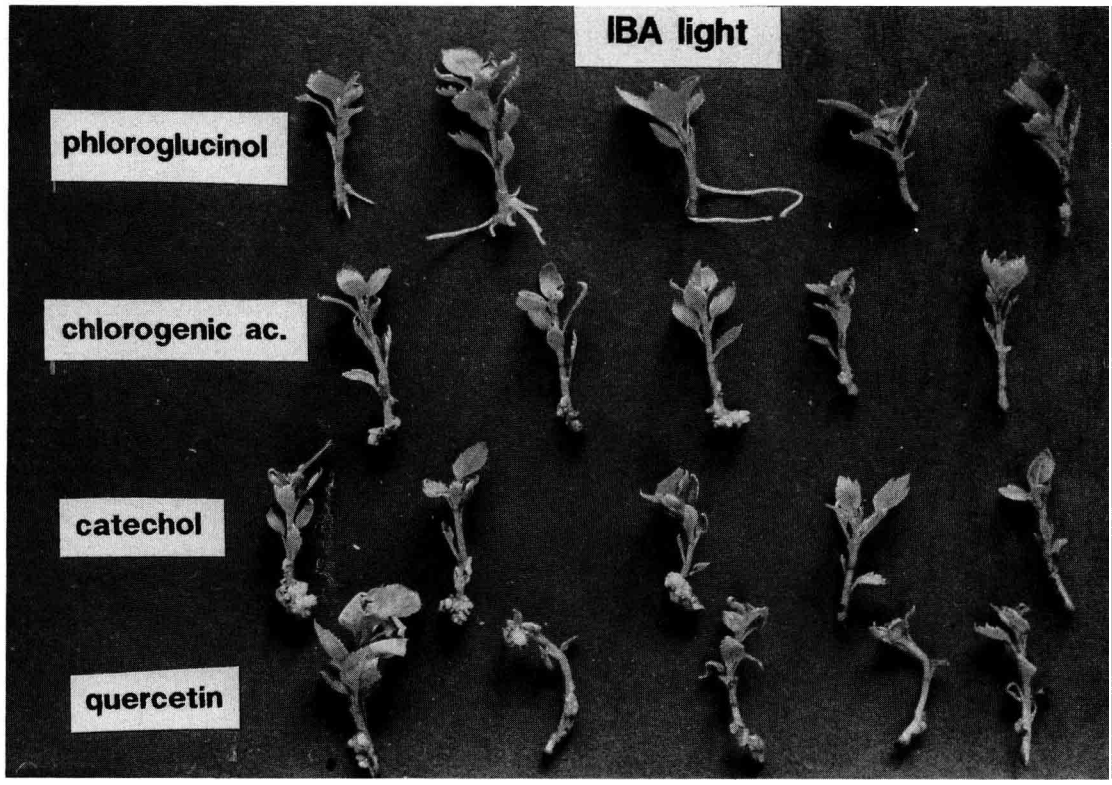

Fig 3. Rooting of M9 Jork with 4 phenols at 1 mM, in the light with IBA 2 mg/l.

Table II. Rooting of M9 Jork: percentage, number of roots per rooted shoot and length of roots (mm) on medium with IBA 2 mg/l and without auxin.

	IBA 2 mg/l			*No auxin*		
	%	*No roots per shoot*	*Length*	*%*	*No roots per shoot*	*Length*
PGL light	60	3.5 ± 0.4	4.5 ± 0.5	40	3.3 ± 0.1	9.3 ± 0.9
PGL dark	25	2.0 ± 0.4	4.0 ± 0.6	35	3.9 ± 0.1	6.0 ± 0.2
CGA light	25	ND	*	25	2.6 ± 0.2	12.9 ± 0.4
CGA dark	30	ND	*	10	1.0 ± 0.1	20.0 ± 1.0
Catechol light	40	ND	*	15	2.2 ± 0.3	11.6 ± 2.0
Catechol dark	25	ND	*	0	–	–
Quercetin light	25	ND	*	10	1.0 ± 0.3	4.0 ± 0.6
Quercetin dark	10	ND	*	0	–	–
Control light	30	9.5 ± 0.8	5.6 ± 0.9	0	–	–
Control dark	15	5.5 ± 1.2	3.1 ± 1.0	0	–	–

ND: not determined as the roots were too short; * non developed roots.

Table III. Multiplication rate of M9 Jork during a subculture (20 d) with different carbohydrate types.

Sucrose + sorbitol (control)	2.7 ± 0.2
Sucrose + galactose	2.7 ± 0.4
Sucrose + maltose	2.5 ± 0.4
Sucrose + fructose	2.2 ± 0.4
Sucrose + glucose	2.2 ± 0.3

Peroxidase activity

Analyses of peroxidase activity were made during the rooting process. The addition of IBA had practically no effect on peroxidase activity (fig 5). The addition of PGL, which increased rooting, resulted in a lower peroxidase activity with respect to other phenols such as catechol and *p*-coumaric acid and the use of IBA alone (figs 5, 6). The electrophoretic patterns did not show particular polymorphism due to the treatments.

DISCUSSION AND CONCLUSION

The satisfactory results for rooting obtained in the light are in contrast with the observations of other authors (Zimmerman, 1984; Zimmerman and Fordham, 1985), who found a beneficial effect due to a short period of growth in darkness. In our experiments the shoots rooted in the light show less callus formation than those rooted after a short period in darkness; this fact could be explained by the presence of riboflavin, which accelerates the photodegradation of IBA (van der Krieken *et al,* 1992).

PGL seems to be effective only when added in the rooting medium: other observations on the use of this phenol during the multiplication phase showed a negative effect on rooting (Damiano *et al,* 1991). Other phenols used in the present experiments did not show the same synergic effect with IBA as PGL did. This confirms the results of Jones and Hatfield (1976), who also found catechol to be less effective than PGL. Regarding certain positive effects of PGL and other phenols in the absence of exogenous

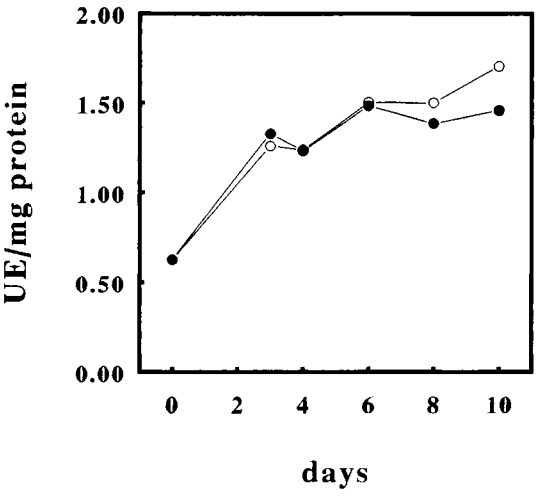

Fig 5. Rooting of M9 Jork, peroxidase activity during rooting without auxin (—O— control) or with IBA 2 mg/l (—●—) in the light.

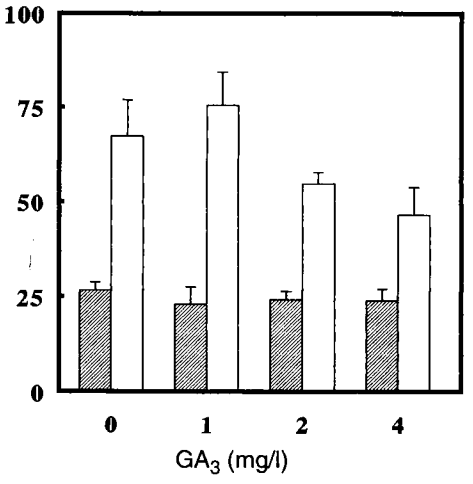

Fig 4. Multiplication rate (x 10) (▨) and rooting percentage (□) with IBA 2 mg/l, PGL 162 mg/l in the light, after subculture with 3 dosages of GA_3.

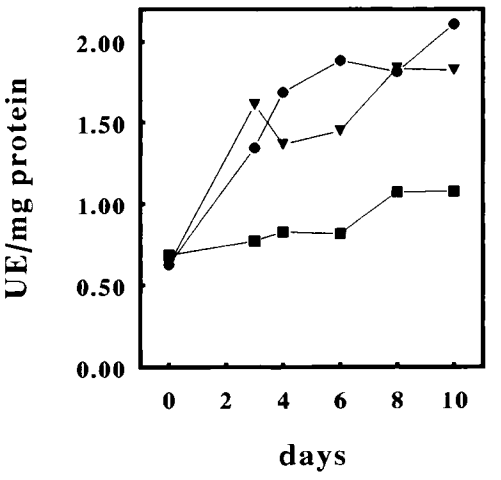

Fig 6. Rooting of M9 Jork, peroxidase activity during rooting with 3 phenols 1 mM with IBA 2 mg/l in the light; —●— catechol; —▼— *p*-coumaric; —■— PGL.

auxins, it may be suggested that in our case the endogenous auxin level was probably increased by the presence of these phenols.

The GA_3 treatments provided contradictory results. After the experiments by Brian *et al* (1960), many other results have confirmed the inhibitory effect of GA_3 on rooting. However, the effects seem to be correlated with the time of application; in fact, GA_3 regulates the activity of IAA-oxidase and therefore controls the endogenous auxin level (Gaspar *et al,* 1977). The effect on rooting ability is less clear when GA_3 is applied during the multiplication phase, although we found a small positive effect at the lowest concentration used.

Our findings show that peroxidase activity without auxin is similar to that with IBA. We also found no clear peaks: peroxidase activity did not decrease. Both sets of data seem to be in contrast with the results of Gaspar *et al* (1990). In this regard it may be advisable to extend the investigation to the first days of rooting, indicated by Gaspar as the critical period for the biochemical changes before rooting expression.

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