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Original article

The effect of sucrose on the development of hybrid walnut microcuttings (*Juglans nigra* x *Juglans regia*). Consequences on their survival during acclimatization

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Summary — We studied the effect of sucrose concentration in the root-development medium on the formation of adventitious roots and survival of microcuttings during acclimatization in 2 interspecific hybrid walnut (*Juglans nigra* n° 23 x *J regia*) clones. Sucrose increased the rooting percentage (fig 1), the number of adventitious roots (fig 2A) and the dry-matter content (table I) per rooted shoot. These effects were due to the energy properties of sucrose rather than to its osmotic function. High sucrose concentrations in the root-development medium (> 20 $g \cdot l^{-1}$) resulted in a high soluble carbohydrate content in the plantlets (fig 3), mainly located in roots and callus. The 2 clones showed different capacities in rooting and growth. Survival of microcuttings during acclimatization was not directly influenced by the sucrose concentration (fig 5) but was correlated with the number of adventitious roots (fig 6A) as well as with the number of leaves (fig 6B) present at the time of transfer to the growth chamber for each individual plant.

walnut / rooting / sucrose / micropropagation / acclimatization / survival

Résumé — Rôle du saccharose sur le développement des microboutures de noyers hybrides (*Juglans nigra* x *Juglans regia*). Conséquences sur leur taux de survie en acclimatation. *Nous avons étudié l'effet de la concentration en saccharose dans le milieu de développement racinaire sur la formation de racines adventives et la survie de 2 clones d'hybrides interspécifiques de noyer* (Juglans nigra *23 x* Juglans regia*). Les expériences montrent que l'enracinement nécessite la présence de saccharose dans le milieu (fig 1). Le saccharose augmente à la fois le taux d'enracinement, le nombre de*

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racines adventives (fig 2A) et la quantité de matière sèche (tableau I) par pousse enracinée. Cet effet du saccharose est à relier à ses propriétés énergétiques plus qu'à son rôle osmotique. Les concentrations élevées en saccharose dans le milieu de développement (> 20 g•l^{-1}) induisent aussi une teneur élevée en sucres solubles dans les pousses enracinées (fig 3), essentiellement des racines et du cal. Les 2 clones montrent des différences d'enracinement et de croissance. La concentration en saccharose dans le milieu de développement n'a pas d'effet sur la survie des microboutures pendant la phase d'acclimatation. La survie des pousses enracinées des 2 clones augmente avec le nombre de racines adventives (fig 6A) et avec le nombre de feuilles étalées (fig 6B) présentes au moment de la phase de transfert en chambre climatisée.

noyer / enracinement / saccharose / micropropagation / acclimatation / survie

INTRODUCTION

Widespread use of micropropagation to produce hybrid walnut plantlets has been limited by the low survival of shoots cultured *in vitro* during acclimatization. *In vitro* culture conditions considerably alter the morphological and physiological features of plantlets compared to plants grown from seeds. The epicuticle of the leaves is poorly developed and their stomata are not functional (Wardle *et al*, 1979). The cultured plantlets are heterotrophic and, in addition to photosynthesis, their energy requirement is largely covered by the carbohydrates from the culture medium. Numerous studies on asparagus (Hasegawa *et al*, 1973) and rose (Hasegawa, 1980) have shown that certain factors of the *in vitro* culture medium (cytokinins, salt concentration) can affect survival during the acclimatization phase. In *Clematis*, a low carbohydrate concentration in the medium increases survival (Lees *et al*, 1991). Furthermore, it should be noted that a low carbohydrate concentration enhances the photosynthetic ability but that carbohydrates are also required for rooting in rose (Hyndman *et al*, 1982) and apple (Pua and Chong, 1985).

This paper is mainly devoted to study the effect of sucrose concentration in the root-development medium on morphology and survival during acclimatization of hybrid walnut microcuttings.

MATERIALS AND METHODS

Plantlet production

The 2 clones used in this study, D152 and M41, are interspecific walnut hybrids (*Juglans nigra* n° 23 x *J regia*). They were established from 2 different embryonic axes isolated axenically (Jay-Allemand and Cornu, 1986) in 2 hybrid walnuts. The shoots were cultured on DKW gelified medium (Driver and Kuniyuki, 1984) containing 4.4 μM benzyl adenine and 0.005 μM indole butyric acid. The shoots, ranging from 20 to 30 mm in length, were induced to root on DKW medium diluted to 1:4 containing 24.6 μM indole butyric acid and 30 g•l^{-1} sucrose. The cultures were kept in the dark for 5 d. After this phase, the shoots were transplanted on a hormone-free medium, composed of a vermiculite/DKW mixture (5:4 v/v) diluted to 1:4 containing sucrose (Jay-Allemand *et al*, 1992). The sucrose concentrations ranged between 0 and 40 g•l^{-1}. All the experiments were conducted in a growth chamber at 28/25 ± 1°C (day/night) with a 16 h daylength and 40 μmol•m^{-2}•s^{-1} photosynthetically active radiation (PAR). The rooting percentage, the number of roots, the total length of the root system, the number of mature leaves and the height of the rooted plantlets were determined after 3 weeks in the root-development medium before the acclimatization phase.

Acclimatization

The acclimatization phase lasted 28 d and was carried out in a growth chamber. During the first 14 d, humidity was kept high by means of a mist system (Defensor), after which the relative humidity was progressively reduced, reaching 70% at the end of the acclimatization phase. The temperature was 28/25 ± 1°C. The photoperiod was a 16 h day with 50 μmol•m^{-2}•s^{-1} PAR. The rooted plantlets were transferred to a substrate containing vermiculite, sand and potting compost (2:1:1, v/v/v). No fertilizer was used. Survival was measured at the end of the acclimatization phase (28 d). A plant was considered to be acclimatized when new leaves had been formed and if there was no necrotic tissue in the apical bud.

Dry matter and biochemical assays

Five plantlets per treatment were collected at random after 3 weeks in the root-development medium. The plantlets were dissected into roots, callus (base of shoots soaking in the auxin treatment), stem and leaves. These differents parts were frozen immediately in liquid nitrogen and freeze-dried (Frossard and Friaud, 1989). The dry matter content of the different parts of each plantlet was then determined. For each sample, the dry matter was ground in a methanol/chloroform/water (12:5:3 v/v/v) mixture for 30 min at room temperature and centrifuged for 20 min at 12 000 *g*. This step was repeated once more (Dickson, 1979).

The collected and purified supernatants were used for glucose, fructose and sucrose determination. The pellet was treated with 0.02 N NaOH and placed in a water bath at 90°C for 30 min to make the starch soluble. The starch was then hydrolysed to glucose by α-amyloglucosidase. Assays of the soluble sugars (glucose, fructose and sucrose) were performed by the enzyme method described by Boehringer (1984).

Data analysis

The effects of the different concentrations of sucrose on the morphology of rooted plantlets for each clone were analysed by a multiple comparison test of the means (Newman and Keuls test). The results expressed as a percentage (rooting and survival) were compared with a χ^2-test. The relationships between plant morphology and survival were determined by the corelation coefficient of Spearman (R_s).

RESULTS

Effect of sucrose on development of the explant

Sucrose concentration in the root-development medium affected the rooting percentage (fig 1). In both clones, rooting increased strongly at 10 g•l^{-1} sucrose as compared to medium without sucrose (+46% in D152 and +59% in M41). When the concentration of sucrose was increased from 10 to 40 g•l^{-1}, no significant change in rooting percentage was observed in clone M41 whereas it rose by 41% in D152. The highest rooting percentage for both clones was obtained on medium containing 40 g•l^{-1} sucrose. Sucrose is also required in the root-development medium for the promotion of root number (fig 2A). There was a 2.6- and 4.8-fold increase in the number of roots per rooted shoot cultured at 30 g•l^{-1} for clones M41 and D152, respectively, compared with shoots cultured without sucrose. Thus, the root number formed per hybrid walnut shoot varies according to the clone and the sucrose concentration.

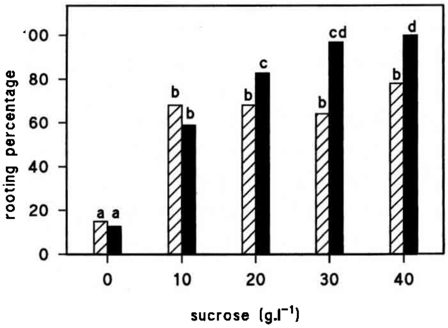

Fig 1. Effect of sucrose concentration in the root-development medium on the rooting percentage of 2 hybrid walnut (*J nigra* n° 23 x *J regia*) clones M41 and D152 after 21 d (D152 ■ *n* = 34, M41 ▨ *n* = 36). Values followed by the same letter are not significantly different (χ^2 test at the α = 5% level).

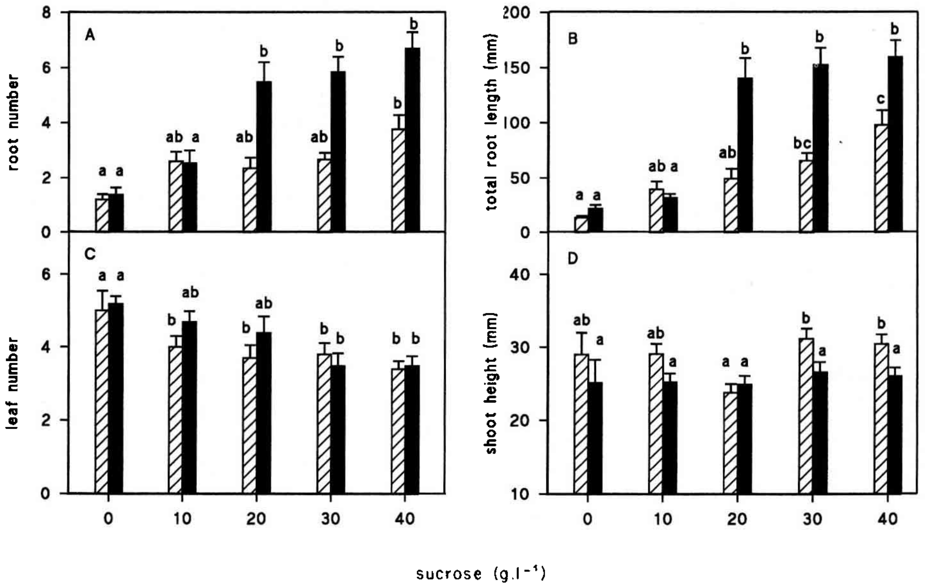

Fig 2. Effect of sucrose concentration in the root-development medium on the average root number (**A**), the total length of the roots (**B**), the average leaf number (**C**) and height (**D**) per rooted plantlet of 2 hybrid walnut (*J nigra* n° 23 x *J regia*) clones M41 and D152 after 21 d (D152 ■, M41 ▨). Values followed by the same letter are not significantly different ($\alpha = 5\%$ average root, total length root and height, $\alpha = 10\%$ leaf number). Results are shown with SE bars.

High sucrose concentrations in the root-development medium significantly increased the length of the root system (fig 2B). Stem length was not affected by variations in sucrose concentration (fig 2D), being 29 and 26 mm for D152 and M41, respectively. The number of mature leaves per rooted shoot decreased in both clones with high sucrose concentrations (fig 2C).

The dry matter increased in the 2 hybrid walnut clones cultured on a medium containing 30 g•l^{-1} sucrose and was twofold greater than on a medium without sucrose (table I). Dry matter accumulated mainly in the roots and the callus of the plantlets cultured on the medium with a concentration of 10 g•l^{-1} of sucrose and higher. The shoots of the clone M41 cultured on a medium containing 40 g•l^{-1} sucrose accumulated 65% of dry matter in the callus whereas it represented only 51% in the clone D152 cultured in the same conditions.

Effect of sucrose in the medium on plant carbohydrate content

The increase in dry matter was associated with a rise in soluble carbohydrate content (glucose, fructose and sucrose) within the plantlet (fig 3), mainly in the roots, although clone M41 also accumulated soluble carbohydrate in the callus. The soluble carbohydrate content of the aerial structure (stem and leaves) seemed to be less affected by the sucrose concentration in the root-development medium than the root system.

Table I. Effect of sucrose concentration in the root-development medium on dry matter distribution after 21 d.

Clone	*Sucrose (g•l^{-1})*				
	0	*10*	*20*	*30*	*40*
M41					
Roots	nd	5 ± 1.3^{a}	11 ± 4.6^{ab}	15 ± 0.5^{bc}	22 ± 2.7^{c}
Callus	17 ± 1.1^{a}	26 ± 3.2^{a}	47 ± 6.6^{b}	72 ± 12.3^{c}	84 ± 6.2^{c}
Stem	6 ± 1.1^{a}	8 ± 1.1^{ab}	6 ± 0.8^{a}	11 ± 1.5^{b}	11 ± 1.9^{b}
Leaves	16 ± 2.1^{ab}	13 ± 1.4^{a}	14 ± 2.3^{a}	19 ± 1.4^{b}	13 ± 1.3^{a}
Plantlet	39 ± 3.4^{a}	52 ± 4.7^{a}	79 ± 9.1^{b}	117 ± 10.5^{c}	130 ± 8^{c}
D152					
Roots	nd	2 ± 0.9^{a}	14 ± 3.2^{ab}	28 ± 2.6^{b}	29 ± 10.1^{b}
Callus	16 ± 1.8^{a}	36 ± 3.1^{ab}	38 ± 4.0^{b}	44 ± 3.7^{b}	47 ± 6.4^{b}
Stem	6 ± 0.8^{a}	9 ± 1.2^{a}	7 ± 0.6^{a}	7 ± 0.6^{a}	8 ± 0.9^{a}
Leaves	11 ± 2.7^{a}	10 ± 1.7^{a}	12 ± 3.5^{a}	7 ± 0.9^{a}	9 ± 1.9^{a}
Plantlet	33 ± 4.5^{a}	57 ± 3.4^{ab}	72 ± 8.1^{b}	86 ± 3.6^{b}	92 ± 17.9^{b}

Values followed by the same letter are not significantly different ($n = 5$, $\alpha = 5\%$). Mean ± SE are indicated. It was not possible to determine root dry matter content for the sucrose-free treatment.

The starch content of the plantlets was generally low (fig 4) except in the callus of clone M41. However, because of a strong increase of dry matter in the callus, the amount of soluble carbohydrates in the shoots of clone M41 was greater than that in D152 shoots (twice as high as from a concentration of 20 g•l^{-1} sucrose).

Relationship between sucrose, morphological features and survival

The survival of the rooted shoots did not differ according to sucrose concentrations in the root-development medium (fig 5). However, it was variable (from 60 to 100%).

An improved survival may have been related to certain morphological features, in particular the number of adventitious roots (fig 6A) and the number of leaves (fig 6B) at transplanting. Irrespective of the sucrose concentration in the development medium, the plantlets of clone M41, with more than 4 roots, had a survival rate of 94%, whereas only 63% of those with fewer than 2 roots survived. The same pattern was observed in clone D152. The correlation coefficient (R_s) between survival and the number of adventitious roots was 0.21 and 0.22 for clones M41 ($n = 61$) and D152 ($n = 74$), respectively, with $a = 5\%$. There was also a good correlation between the survival of rooted shoots and the number of adult leaves at transplanting (M41, $R_s = 0.24$, D152, $R_s = 0.45$ with $a = 5\%$).

DISCUSSION

Sucrose in the root-development medium is one of the major factors for both obtaining a high rooting percentage and promoting root elongation. The role of sucrose in rooting is more closely linked to the energy supplies than to its osmotic properties, as observed by Hyndman *et al* (1982) in rose shoots. When sucrose was replaced in the development medium by mannitol (which has the same osmotic potential, $p = 2.2$ bar), the shoots of clone D152 did not form roots (unpublished results). This finding is simi-

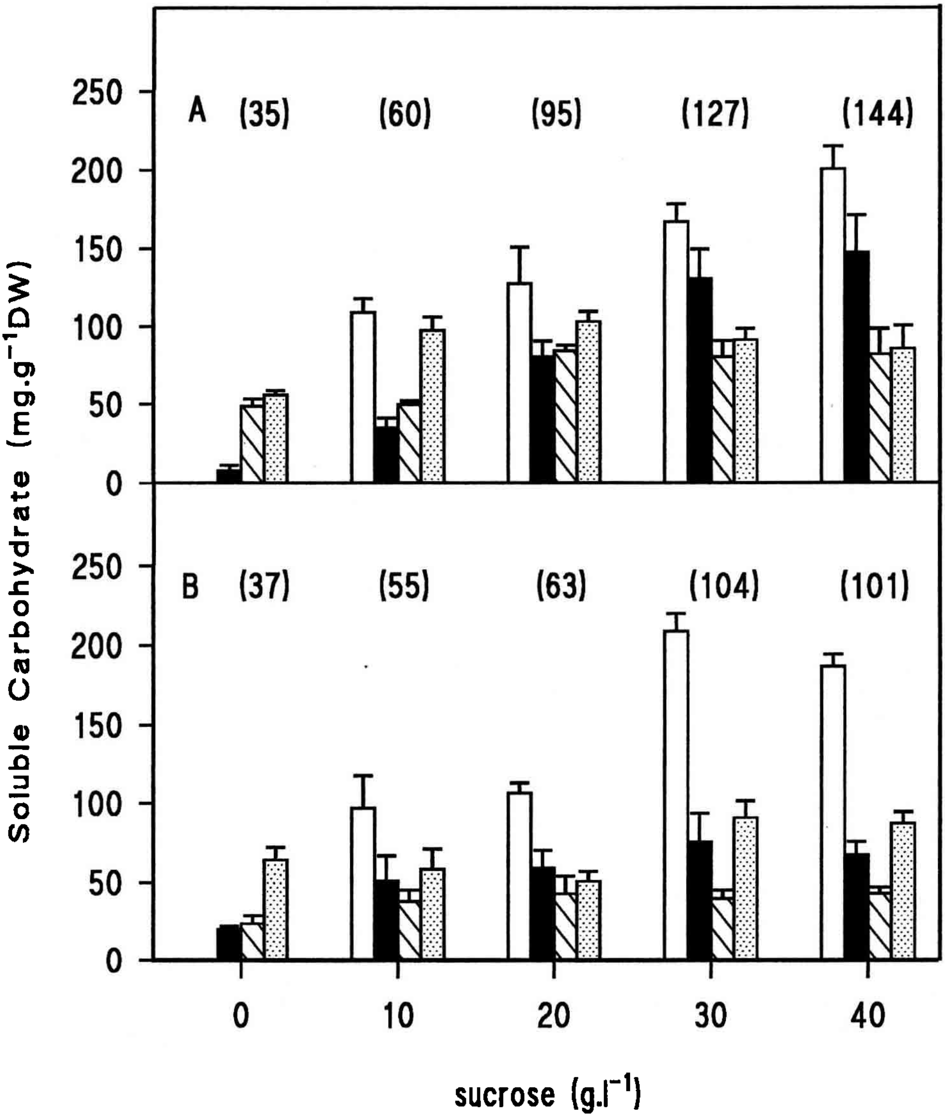

Fig 3. Effect of sucrose concentration in the root-development medium on the soluble carbohydrate content: glucose + fructose + sucrose in the differents parts of the plantlet roots □, callus ■, stem ▧ and leaves ▒ of 2 hybrid walnut (*J nigra* n° 23 x *J regia*) clones M41 (**A**) and D152 (**B**) after 21 d. The values between brackets are the soluble carbohydrate contents of the whole plantlet (mg•g^{-1} DW). Results are shown with SE bars ($n = 5$).

lar to the observations of Greenwood and Berlyn (1973), who also showed that sucrose could not be replaced by osmotic agents such as mannitol or methyl-α-D-glucopyranoside in *Pinus lambertiana* cuttings. The energy requirements of *in vitro* plants are mainly covered by the sucrose taken up from the medium. Therefore, it can be

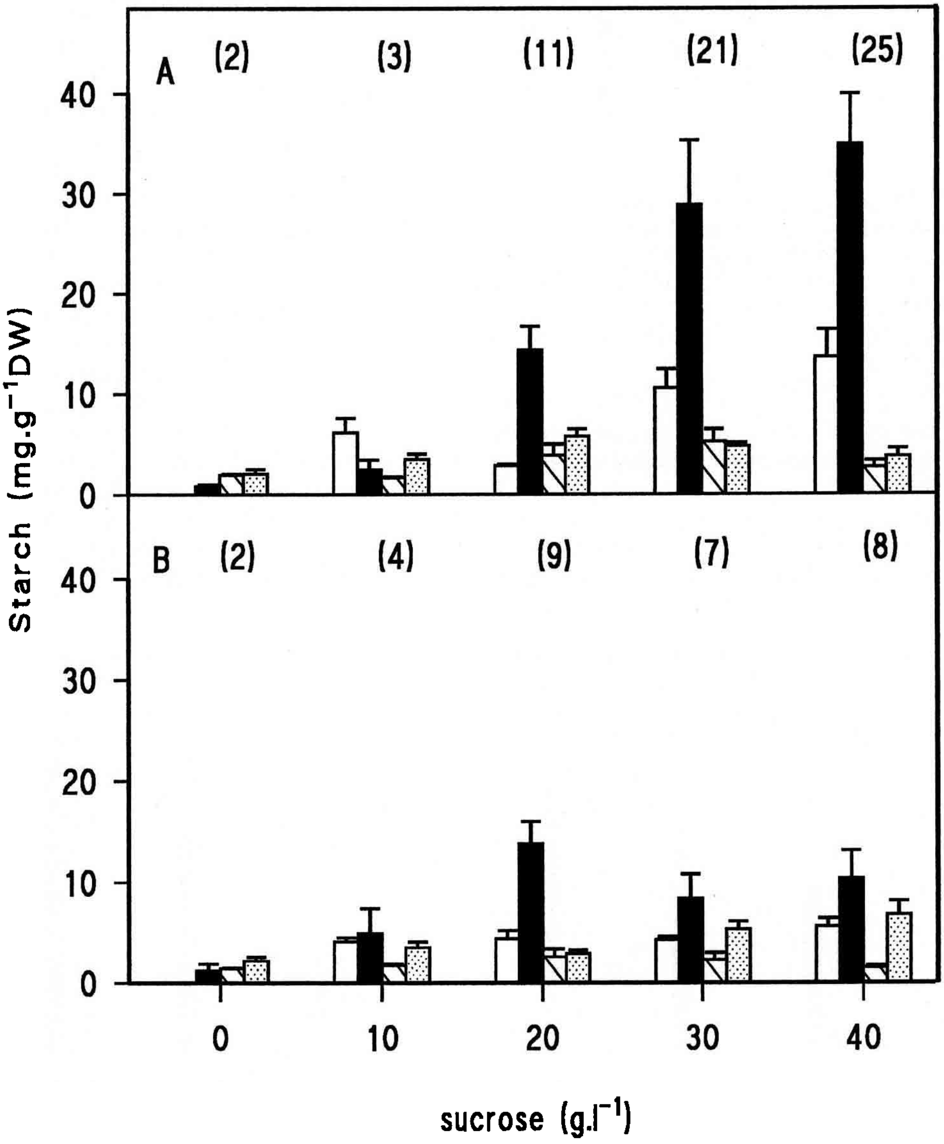

Fig 4. Effect of sucrose concentration in the root-development medium on the starch content in the different parts of the plantlet roots □, callus ■, stem ▧ and leaves ▒ of 2 hybrid walnut (*J nigra* n° 23 x *J regia*) clones M41 (**A**) and D152 (**B**) after 21 d. The values between brackets are the starch contents of the whole plantlet (mg•g^{-1} DW). Results are shown with SE bars (n = 5).

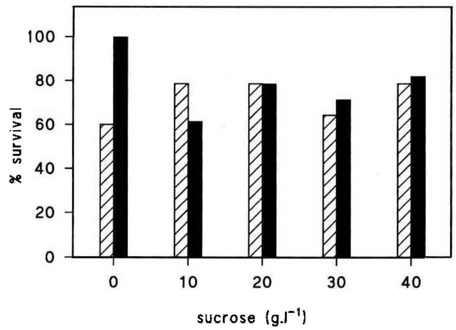

Fig 5. Effect of sucrose concentration in the root-development medium on the survival of rooted planted of 2 hybrid walnut (*J nigra* n° 23 x *J regia)* clones after 28 d of acclimatization (D152 ■ and M41 ▨ , $n = 14$ except 0 g/l, $n = 5$). No significant difference was observed with χ^2 test at the $\alpha = 5\%$ level.

suggested that the assimilates produced by photosynthesis are not sufficient to meet the energy requirements of the root primordia, which are very high for the initiation and development of the organs. This hypothesis is supported by the results of a previous study (Thorpe and Meier, 1972). The authors observed that shoot formation in tobacco callus increased respiration as the result of the use of carbohydrate reserves in the callus as starch. Indeed, we observed an antagonism between the number of roots and the number of leaves in the walnut plantlet.

We observed that the hybrid walnut plantlets cultured on a medium with a high sucrose concentration had a high soluble carbohydrate content. It was similar to that observed in potato plantlets (Cournac *et al,* 1991) and in hop plants (Howard and Sykes, 1966). The high sucrose concentration also led to high dry matter accumulation in walnut plantlets but all plantlet parts were not affected similarly. The dry matter accumulation was greater in roots and callus than in the aerial part. Mousseau (1986) reported the same difference in dry matter accumulation in tobacco cultured *in vitro* with or without sucrose. In this species, the dry matter decrease in plantlets cultured without sucrose was not compensated for by CO_2 enrichment of the atmosphere.

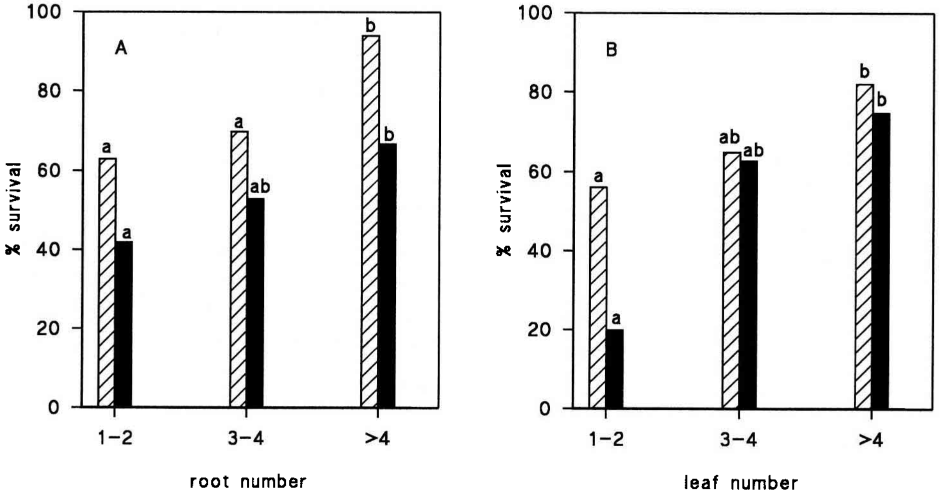

Fig 6. Relationship between the survival of rooted plantlets of 2 hybrid walnut (*J nigra* n° 23 x *J regia*) clones M41 ▨ and D152 ■ and initial number of roots (**A**) or initial number of leaves (**B**) after 28 d of acclimatization. Values followed by the same letter are not significantly different (χ^2 test at the $\alpha = 5\%$ level).

The roots and the callus were favourable sites for the accumulation of soluble carbohydrates. The starch content was low compared to the soluble carbohydrate contents. Capellades *et al* (1991) observed that the unrooted rose shoots grown on a high sucrose concentration accumulated starch in the chloroplast and showed the highest survival rate during acclimatization. The low starch content of the rooted shoots could be due to a high degradation of carbohydrates by an intense respiratory metabolism devoted to the root growth. The good correlation between the number of roots per rooted shoot and survival of hybrid walnut could be explained by the carbohydrates stored in the roots and callus. These reserves were used during the acclimatization. Moreover, a well-developed root system improves water absorption and salt nutrition during acclimatization. The larger root system of Douglas fir plantlets absorbed more water and increased photosynthetic activity (Mohammed and Vidaver, 1991).

Despite a significant difference between the number of roots and the sucrose concentration in the root-development medium, no relationship was observed between the sucrose concentration and the survival of *in vitro* walnut hybrids. Thus, other factors must be involved in the survival of rooted shoots.

The number of mature leaves at transplanting seems to be an important morphological criterion in the survival of rooted shoots of hybrid walnut clones. The number of mature leaves is an indicator of both the ability of the apical bud meristem to produce new leaves during the acclimatization phase and the photosynthetic activity of leaf area. During acclimatization, the plantlet has moved up from mixotrophic status to autotrophic status. Plant autotrophy depends effectively on the appearance of new leaves adapted to the new environmental conditions (Donnelly and Vidaver, 1984). The autotrophic status in cauliflower plantlets is only established from the second week of transfer (Grout and Aston, 1978). The growth of new organs adapted to acclimatization conditions is promoted by energy either from photosynthesis or from carbohydrates stored in the plantlet during the root-development phase.

Therefore, the survival of plantlets depends on the root system and on the stage of development of the aerial structure. The development and the morphology of the aerial structure at transplanting played a more important role than that of the root system, as previously observed in eucalyptus clones (Poissonnier *et al*, 1983) and Loblolly pine (Wisniewski *et al*, 1986). Madec *et al* (1979) noted that the absence of leaves was the main reason for mortality during acclimatization.

It has been observed that a low sucrose concentration in the propagation medium enhances photosynthetic ability of rose shoots (Langford and Wainwright, 1987) and consequently the establishment of photoautrophy (Leclerc and Creche, 1991) during the tissue culture. The assimilation rate observed on plantlets grown on a medium without sucrose was similar to that of seedlings (Short *et al*, 1987). In hybrid walnut, the absence of sucrose in the root-development medium induced a low rooting percentage, which is a real problem. Further studies are necessary to determine whether both the CO_2 enrichment in the vessel and the high light intensity in the growth chamber stimulate root growth and autotrophy on the sucrose-free medium of the hybrid walnut plantlets.

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