Factors affecting shoot multiplication and rooting of walnut (*Juglans regia* L.) in vitro

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Abstract

***Juglans regia* is one of the most important nut crops rich in oil, protein and vitamins, and its high value timber is used for furniture, veneer and gunstock production. However, the propagation of *J. regia* is strongly dependent on the genotype. Slow in vitro growth results in low propagation rates and difficulties are encountered using conventional rooting methods. Embryo axes dissected from mature nuts were used as primary explants for establishing shoot cultures. The growth of walnut shoot cultures was compared on media differing in nutrient formulation (Rugini olive medium and DKW medium), concentrations of 6-benzyladenine (BA) (2.2-8.8 µM), solid and liquid medium and indole-3-butyric acid (IBA) concentration (4-20 µM). During the 21 days culture passage the Rugini medium gave significantly higher fresh mass and numbers of axillary shoots than DKW medium. The best BA concentration for *J. regia* explants was 2.2 µM BA. The results also indicated that solid medium (7 g L^{-1} agar) was better than liquid medium for shoot proliferation. The optimal IBA concentration in the induction medium for rooting of microcuttings was 12 µM IBA applied for 5 days in darkness before transfer to root expression media. There was no significant difference among two root expression media (¼ DKW + Vermiculite and ¼ MS + Vermiculite) in rooting of microcuttings after 21 days. The acclimatization of rooted microcuttings was difficult because the tendency to dessicate of plantlets and because of the occurrence of diseases due to high humidity in the greenhouse. Nevertheless, 80% of the *J. regia* plantlets were successfully established in the greenhouse.**

Keywords: benzylaminopurine, DKW medium, indole-3-butyric acid, root induction, root expression, Rugini olive medium

INTRODUCTION

Walnut (*Juglans regia* L.) is one of the world's most valuable tree species for nut and wood production. Nuts are a rich source of oil, protein, vitamins and their wood is used for furniture, veneer, and gunstock production with high commercial value. However, the main problems with in vitro propagation are long propagation cycles and low rooting percentages. (Cornu and Jay-Allemand, 1989; Leslie and McGranahan, 1992; Toosi et al., 2010) .

The aims of this study were to evaluate different culture media varying in nutrient composition and cytokinin concentration, to compare between solid and liquid medium, to identify the best IBA concentration in the root induction phase and to find out the best basal medium in the root expression phase for *J. regia* microcuttings.

MATERIAL AND METHODS

Nuts of Persian walnut (*J. regia* L.) were harvested in Hann. Münden, Germany. The epicarp was removed and the nuts were disinfected for 2 min in 70% ethanol, then for 20 min in 5% NaOCl with several drops of Tween and finally washed in sterile distilled water. Embryo axes were isolated from the nuts under sterile conditions and quickly dipped in

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70% ethanol followed by immersion in 0.5% NaOCl with several drops of Tween 20 for 5 min. The embryo axes were rinsed two times in sterile water and placed on DKW medium with 4.4 µM 6-benzyladenine (BA) and 0.2 µM indole-3-butyric acid (IBA) (Payghamzadeh and Kazemitabar, 2010) (Figure 1a and b).

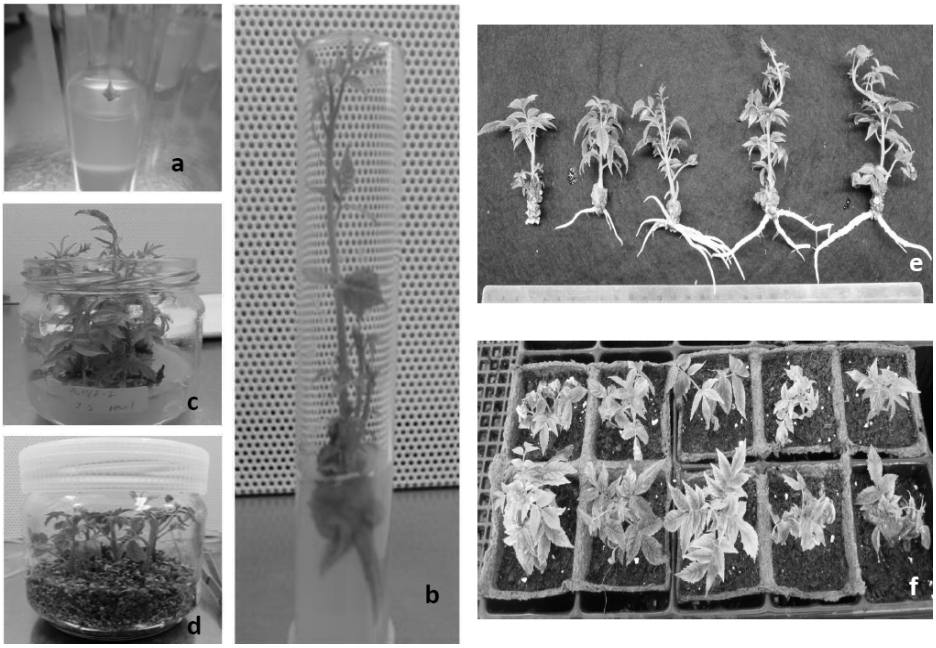

Figure 1. Micropropagation of *Juglans regia*. (a) Embryo axes; (b) Germinating embryo axes; (c) Propagation phase; (d) Rooting with vermiculite medium; (e) In vitro rooted shoots of *J. regia,* (f) Plantlets of *J. regia* established in the greenhouse.

All media were sterilized by autoclaving at 121°C and 1.0 kg cm^{-2} pressure for 20 min. The pH was adjusted to 5.7 prior to autoclaving. Cultures were maintained in a growth chamber under a 16-h photoperiod and temperatures of 27±1°C under cool white fluorescent lamps at 75 µmol m^{-2} s^{-1}.

Shoot cultures of one established genotype were used for the evaluation of two basal media (pre-prepared Duchefa), DKW medium (Driver and Kuniyuki, 1984) and Rugini olive medium (Rugini, 1984) (Table 1).

The cytokinin BA was supplied in three concentrations of 2.2, 4.4 and 8.8 µM. Glucose was used at 30 g L^{-1} and Kobe agar (Serva Co.) at 7 g L^{-1}. The shoots were cultured in 250 mL glass jars containing 80 mL of solidified medium. At the end of the experiment (after 21 days) the following parameters were measured: callus fresh mass, shoot fresh mass and main shoot length and number of axillary buds. Each experiment consisted of a completely randomized design with five replicates, each replicate containing five explants (Figure 1c).

In the following experiment, the best culture medium from the first experiment was used (Rugini medium with 2.2 µM BA). In the multiplication phase, shoots of *J. regia* were cultured in solid (7 g L^{-1} Kobe agar) and liquid medium (without agar) by the static conventional system and the explants were inserted vertically to the depth of a net above the liquid medium. The parameters evaluated were the same as described above.

According to the technique described by Jay-Allemand and Cornu (1986) for rooting of *J. regia*, two phases were assessed: (1) Induction phase: microcuttings were placed on root induction medium in the dark at 27°C for 5 days before transferring to root expression medium. The induction medium was supplemented with 4 µM, 12 µM and 20 µM IBA, respectively, 30 g L^{-1} glucose and 7 g L^{-1} Kobe agar (2) Expression phase: after the comparison between the three IBA concentrations, two root expression media (¼ DKW + Vermiculite and ¼ Murashige and Skoog (1962, MS) + Vermiculite) were compared. The parameters evaluated were main root length and root number per rooted shoot.

Table 1. Composition of the tissue culture media used for walnut shoot multiplication. Source: Driver and Kuniyuki (1984) and Rugini (1984).

Nutrient	Culture media	
	DKW	Rugini olive medium
Macronutrients	(mg L^{-1})	(mg L^{-1})
NH_4NO_3	1416.00	412.00
$CaCl_2$	112.50	332.16
$Ca(NO_3)_2 \times 2H_2O$	1664.64	-
$Ca(NO_3)_2$	-	416.92
KH_2PO_4	265.00	340.00
K_2SO_4	1559.00	-
KNO_3	-	1100.00
$MgSO_4$	361.49	732.60
KCl	-	500.00
Micronutrients		
H_3BO_3	4.80	12.40
$CuSO_4 \times 5H_2O$	0.25	0.25
$MnSO_4 \times H_2O$	33.80	16.90
$Na_2MoO_4 \times 2H_2O$	0.39	0.25
$ZnSO_4 \times 7H_2O$	17.00	14.30
KI	-	0.83
$CoCl_2 \times 6H_2O$	-	0.025
FeNa EDTA	44.63	36.70
Vitamins		
Nicotinic acid	1.00	5.00
Thiamine HCl	2.00	0.50
Pyridoxine	-	0.50
Myo-inositol	100.00	100.00
Biotin	-	0.05
Folic acid	-	0.50
Glycine	2.00	2.00

For the acclimatization process, plantlets of *J. regia* from in vitro rooting (from both ¼ DKW + Vermiculite and ¼ MS + Vermiculite expression medium) were directly inserted in a mix of peat: perlite (1:1) with an intermittent fog system. The humidity in the greenhouse was 100% from the 1st to the 14th day, followed by 90% from the 15th to the 21st day. The temperature was 24/21°C (day/night). The survival of the plantlets was assessed after 21 days.

The results were analyzed according to analysis of variance, and separation of means was tested using Tukey's HSD (Honestly Significant Difference) test, at the level $P<0.05$ by the Statistica 12 (StatSoft) program.

RESULTS AND DISCUSSION

Our data indicate that both DKW and Rugini medium, supplemented with the lowest BA concentration of 2.2 µM resulted in a significantly higher main shoot length and a higher number of axillary buds (4.3 and 4.5 cm, 7.9 and 11.7 buds, respectively) after 21 days of culture (Table 2). Based on mean values, the most appropriate culture medium and cytokinin concentration for the in vitro multiplication appeared to be Rugini medium with 2.2 µM BA (Figure 2). Thus, differential nutrient content in the culture media (Rugini medium contains more Ca^{2+} and K^{+} but less NH_4^{+} and SO_4^{2-} than DKW medium) may have a significant effect on shoot development. This finding is consistent also with other investigations (see e.g., Šedivá et al., 2013 on horse chestnut, *Aesculus hippocastanum*).

Table 2. Growth of *J. regia* explants after 21 days of culture in different nutrient media and concentrations of BA.

Nutrient medium (µM)	Callus fresh mass (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
DKW				
2.2 BA+0.2 IBA	1.19±0.79a[1]	0.45±0.21a	4.34±1.00a	7.88±2.67a
4.4 BA+0.2 IBA	1.96±1.01b	0.59±0.56ab	3.20±1.84b	7.76±3.38a
8.8 BA+0.2 IBA	1.97±1.26b	0.27±0.28a	1.20±0.99c	3.92±3.04b
Rugini				
2.2 BA+0.2 IBA	1.38±0.62a	0.52±0.19a	4.49±0.99a	11.72±3.5a
4.4 BA+0.2 IBA	2.18±0.78b	0.66±0.30ab	3.70±1.53a	9.44±2.41b
8.8 BA+0.2 IBA	1.20±0.52a	0.44±0.24a	1.54±1.23b	8.00±3.51b

[1]Values followed by the same letter within the same column (and nutrient medium) are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

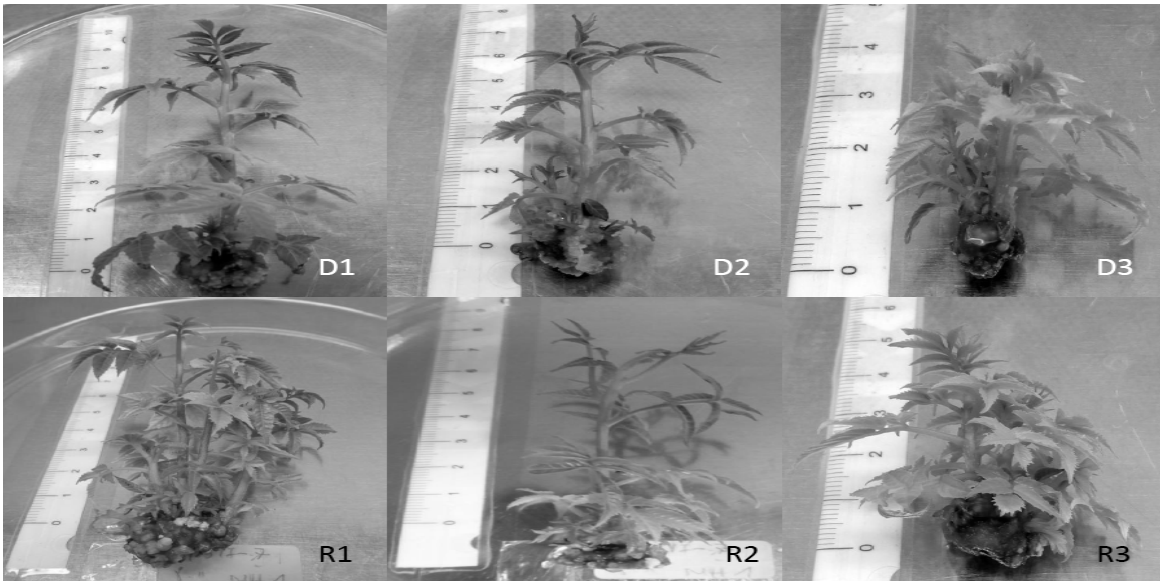

Figure 2. *J. regia* explants after 21 days of culture in different nutrient media and concentrations of BA. D1-3: DKW medium with 2.2 µM BA + 0.2 µM IBA (D1), 4.4 µM BA+ 0.2 µM IBA (D2) and 8.8 µM BA + 0.2 µM IBA (D3). R1-3: Rugini medium with 2.2 µM BA + 0.2 µM IBA (R1), 4.4 µM BA + 0.2 µM IBA (R2) and 8.8 µM BA + 0.2 µM IBA (R3).

Furthermore, significant differences were found for growth parameters of the *J. regia* explants after 21 days when using solid and liquid Rugini medium containing 2.2 µM BA + 0.2 µM IBA (Table 3). More axillary buds and longer shoots were obtained in the solid medium with 7 g L^{-1} agar than in liquid medium (9.2 against 5.4 buds and 3.3 against 2.1 cm shoot length, respectively). However, we could observe that the shoots in liquid medium appeared fresher and greener than in solid medium and hyperhydricity was observed on shoots in liquid medium. This is in accordance with observations on *Dianthus caryophyllus* (carnation) and *Cynara cardunculus* var. *scolymus* (artichoke): hyperhydricity may be a result of too high humidity in the medium or of growth on a liquid medium (Debergh et al., 1981).

Table 3. Growth of *J. regia* explants after 21 days of culture in liquid and solid Rugini medium with 2.2 µM BA and 0.2 µM IBA.

Gelling agents	Callus mass fresh (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
7 g L^{-1} agar	0.99±0.46a[1]	0.46±0.19a	3.27±0.19a	9.24±3.14a
No agar	0.47±0.27b	0.26±0.16b	2.12±0.39b	5.40±2.78b

[1]Values followed by the same letter within the same column are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

Following the two-phase of rooting procedure described above, the rooting rate on ¼ DKW + Vermiculite expression medium was higher (56%) than on ¼ MS + Vermiculite expression medium (47%) (Figure 3) after 21 days and there was no significant difference in main root length in ¼ DKW + Vermiculite and ¼ MS + Vermiculite expression medium (Table 4). However, in ¼ DKW+ Vermiculite following induction with 12 µM IBA the significantly highest root number per rooted shoot of 2.1 was obtained, whereas the differences observed on ¼ MS + Vermiculite expression medium were not significant for all three IBA concentrations applied during induction (Table 4; Figure 1d, e).

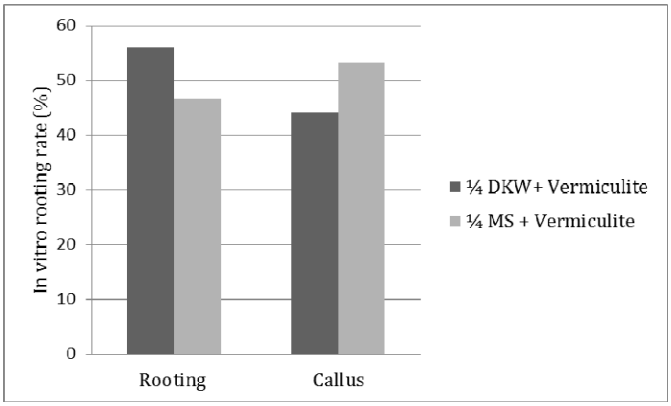

Figure 3. In vitro rooting rate of *J. regia* microcuttings after 21 days in expression medium.

Table 4. Effect of IBA concentration in the induction medium and of nutrient composition in the expression medium on rooting of *J. regia* microcuttings after 21 days.

Rooting medium	Main root length (cm)	Root number per rooted shoot
¼ DKW + Vermiculite (expression)		
4 µM IBA (induction)	0.80±1.22a[1]	0.52±0.77b
12 µM IBA (induction)	1.33±1.17a	2.08±1.80a
20 µM IBA (induction)	0.41±0.85a	0.56±1.08b
¼ MS + Vermiculite (expression)		
4 µM IBA (induction)	0.80±1.13a	0.68±0.98a
12 µM IBA (induction)	1.52±1.54a	1.48±1.58a
20 µM IBA (induction)	0.91±1.47a	0.52±1.08a

[1]Values followed by the same letter within the same column (/nutrient medium) are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

During acclimatization in the greenhouse more than 80% of the plantlets from in vitro rooting (for both DKW and MS root expression media) continued to grow vigorously (Figure 1f), whereas the rest of the plantlets collapsed after transplanting to the greenhouse. Furthermore, 71.4% of the plantlets with callus continued to grow while 28.6% of the plantlets with callus died immediately or temporarily survived during acclimatization (21 days) without producing any further growth (Figure 4).

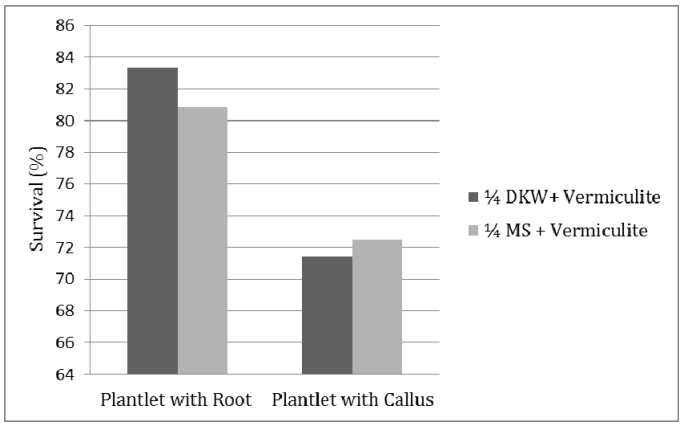

Figure 4. Plantlet survival rate of *J. regia* microcuttings after 21 days in greenhouse.

CONCLUSIONS

The use of Rugini medium supplemented with 2.2 µM BA, 0.2 µM IBA and 7 g L^{-1} Kobe agar is suggested for in vitro shoot proliferation of *J. regia* starting from embryo axes. For in vitro rooting, the best IBA concentration in the induction medium was 12 µM IBA for 5 days in darkness before transferring the *J. regia* microcuttings to root expression medium. There was no significant difference among two root expression media (¼ DKW and ¼ MS) after 21 days.

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