DOI : 10.5958/0974-0112.2017.00057.3

Indian J. Hort. 74(2), June 2017: 285-287

Short communication

Technique to minimize phenolics in walnut *in vitro* culture initiation

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ABSTRACT

Total phenol content of the walnut genotypes was determined to explore the relationship between total phenols and regeneration response for different walnut genotypes. Walnut leaves from ten genotypes (CITH-Walnut-I, CITH-Walnut-II, BBW-8, CITH-Walnut-IV, BP-3, SP-1, LG-11, Hamdan, Suleiman, and Opex Culchery) were used for extraction of total phenols through modified Folin-Ciocalteu method. Total leaf phenolic content ranges from 140 µg g^{-1} (WGB-1) to 285 µg g^{-1} (BBW-8). Phenolic interactions expressed as darkening of the explants lead to death. Among different antioxidants used ascorbic acid @ 350 mg/l was found best with almost no phenol exudation in the medium and shoot initiation occurred after 8 days of inoculation. The number of shoots was highest (10), followed by citric acid used @ 350 mg/l showing low degree of exudation where shoot initiation was noted in 15 days and with 15.0 shhots per explant.

Key words: Walnut, phenolic content, antioxidants, *in vitro* culture, ascorbic acid.

Walnut tree (*Juglans regia* L.), is native to Eastern Europe and North Asia, but is also found throughout North, Central and South America. The tree has great socio-economic importance being frequently cultivated in temperate zones of the world mainly because of its edible seed, having oil which is rich in unsaturated fatty acids, phytosterols and tocopherols. For many years the trees are grown in orchards because of the delicious fruits, which can be eaten raw and are excellent for dessert as well as in baking and confectionery. Walnut is propagated through grafting and budding, but survival rate is very low. Micropropagation provides more efficient technique for large scale multiplication. Various attempts have been made using different types of explant, media, culture condition and rooting techniques, with promising results (McGranahan *et al.*, 1; Vahdati *et al*., 2). High phenolic content in explants effect the *in-vitro* multiplication of walnut. Phenolics are involved in growth and reproduction and provide plants with resistance to pathogens and predators. In tissue culture studies, phenolic substances, especially oxidized phenolics generally effect *in-vitro* proliferation negatively. Tissue browning and blackening are also one of the major problems for *in-vitro* culturing in many economically important plants. Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explant. The production of exudates from freshly cultured explants of walnut has also been a problem, solved by employing explant pre-soaks and transferring explants frequently to fresh medium. Frequent sub-culturing and use of some antioxidants such as citric acid, ascorbic acid, PVP (polyvinyl pyrrolidone) and activated charcoal, which can reduce phenolic oxidation and contribute to regeneration from explants (Toth *et al.*, 3). In this study we determined total phenol amount in leaves of different walnut genotypes and best media conditions (anti-oxidant supplement) was standardized for efficient regeneration and shoot multiplication of difficult to regenerate walnut genotype.

Material for analyses was taken from two-year-old ten walnut genotypes (CITH-Walnut-I, CITH-Walnut-II, BBW-8, CITH-Walnut-IV, BP-3, SP-1, LG-11, Hamdan, Suliman, and Opexculchery) maintained in polyhouse of ICAR-Central Institute of Temperate Horticulture, Srinagar, India. For each sample, about 0.5-1.0 g of leaf were manually collected and grinded into mortar and pestle in 80% ethanol. Total phenolic content was determined by using modified Folin-Ciocalteu method in the extracts. An aliquot of the extract was mixed with 5 ml Folin-Ciocalteu phenol reagent method. About 0.5 ml of Folin-Ciocalteu solution was added to each sample and allowed to stand for 3 min. in dark and then 2 ml of 20% Na_2CO_3 was added and mixed thoroughly. All the samples were kept in boiling water bath for 1 min. and absorbance was measured at 650 nm using UV double spectrophotometer. Experiments have been made with axillary buds and nodal segments of walnut. Juvenile material was taken from 1-2 year-old plantlets germinated under greenhouse conditions that had been kept under stringent plant protection conditions. The medium used was DKW

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medium (Driver and Kuniyuki, 4) supplemented with 3% sucrose, 0.7% agar-agar and BAP (2 mg/l), IBA (0.1 mg/l) and GA_3 (0.1 mg/l). The culture conditions were 16 h photoperiod at 25°C. To decrease explant exudation, sub-culturing was done regularly after 10-15 days. To prevent phenolic oxidation, antioxidants like ascorbic acid, citric acid, PVP and activated charcoal. Each treatment was replicated 4 times and observations in stages of development were recorded periodically. The data was analyzed by comparing means using one way ANOVA and the significance was determined by Duncan's Multiple Range Test using SPSS for windows (v. 15. SPSS Inc USA).

Total phenols expressed as percentage of dry weight of leaf material varied significantly among species. BBW-8 and Suleiman had the highest phenol concentrations (285 and 280 µg/g of leaf tissue respectively), whereas WGB-I and Opexcultury leaves had very low levels (140 and 155 µg g^{-1} of leaf tissue respectively). Phenol contents were significantly different among the walnut genotypes (Table 1). Phenol exudation and tissue culture response was directly related to total phenol content. Degree of phenol exudation was again highest in BBW-8 and Suleiman, while as it was lowest in WGB-I (Table 1). Phenol exudation greatly influenced the tissue culture response and was found to be correlated with quality control (Joana *et al.*, 5). Exudation of phenolics has been reported to cause explants necrosis in *in-vitro* culture of many plant species (Standardi and Romani, 6). Negative effect of phenolics on *in-vitro* proliferation has also been revealed by other researchers (Ahmed *et al.*, 7). DKW medium was chosen for this study based on previous studies which revealed that DKW medium is suitable (and in many cases superior) for the culture of *J. regia* as well as other *Juglans* species (Driver and Kuniyuki, 4; Vahdati *et al.*, 2). Mature, dormant seeds of "Hamdan" variety were harvested from CITH, Farm and were stored at 5°C before being used. Seeds were then stratified in moist sand at 5°C in darkness for 90 to 120 days. After stratification, seeds were germinated in plastic pots containing a 1 peat: 1 perlite (v/v) medium. Seedlings were maintained in a temperature controlled polyhouses for 2 to 3 months. Stems from actively growing seedlings were stripped of leaflets and surface disinfested in 0.8% (v/v) sodium hypochlorite (15% Clorox™ bleach) for 15 to 20 min., followed by four rinses in sterile, deionized water. Nodal explants (1 to 2 cm long) were excised and placed upright in 25 × 95-mm culture vials containing 15 ml of DKW medium supplemented with 3% sucrose, 0.7% agar and BAP (2 mg/l), IBA (0.1 mg/l) and GA_3 (0.1 mg/l).

DKW medium gave good results with respect to shoot multiplication in walnut genotypes and the application of 0.1 mg/l GA_3 induced better shoot elongation (Vahdati *et al.*, 2; Ahmed *et al.*, 7). Application of different anti-oxidants to medium greatly influences the regeneration rate and survival of explants. Results showed that the number of axillary shoots arising from the micro-shoots was highest (7.0) on medium supplemented with 350 mg/l ascorbic acid, 5 on medium with 350 mg/l citric acid and 250 mg/l ascorbic acid compared to control (Table 2, Fig. 1). Application of antioxidants also influenced the rate of shoot initiation. Media supplemented with 350 mg/l ascorbic acid took only few days (8) for shoot initiation followed by 13 days on medium with 250 mg/l ascorbic acid (Table 2). Role of antioxidants in improving tissue culture response has been reported by number of workers (Vahdati *et al.*, 2; Ahmed *et al.*, 7). The addition of ascorbic acid

Table 1. Differential response of walnut genotypes in tissue culture with respect to different phenolic levels.

Genotype	Total phenolics (µg g^{-1})	Degree of phenolic exudation
Sulieman	280^{f} ± 0.82	++++
Hamdan	215^{d} ± 2.04	++++
LG-5	165^{c} ± 2.04	+++
LG-9	170^{c} ± 2.04	+++
SP-3	235^{e} ± 5.40	++++
BP-4	167^{c} ± 1.91	+++
KPT-5	230^{e} ± 2.04	++++
WGB-1	140^{a} ± 2.04	++
Opex Culchery	155^{b} ± 2.04	+++
BBW-8	285^{f} ± 3.54	++++

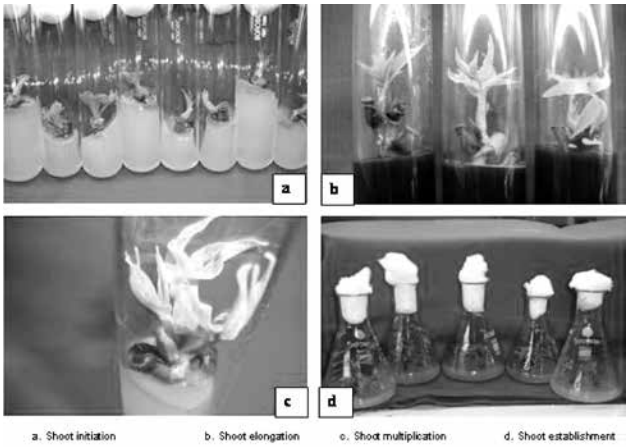

Fig. 1. *In-vitro* multiplication of *Juglans regia*.

Table 2. Effect of different antioxidants on *in vitro* multiplication of walnut genotype "Hamdan".

Antioxidant	Conc. of antioxidant (mg /l)	Degree of phenolic exudation	Days to shoot initiation	No of shoots/ explant
Control	0.0	++++	$27^{h} \pm 0.71$	$2^{a} \pm 0.41$
Ascorbic acid	150	+++	$17^{de} \pm 0.41$	$4^{ab} \pm 0.71$
	250	++	$13^{b} \pm 0.41$	$7^{c} \pm 0.41$
	350	-	$8^{a} \pm 0.41$	$10^{d} \pm 1.08$
Citric acid	150	+++	$22^{g} \pm 0.82$	$4^{ab} \pm 0.41$
	250	++	$18^{ef} \pm 1.41$	$4^{ab} \pm 0.82$
	350	+	$15^{bcd} \pm 0.41$	$8^{cd} \pm 0.71$
PVP	0.1%	+++	$20^{f} \pm 0.82$	$3^{a} \pm 0.41$
	0.2%	+++	$17^{de} \pm 0.41$	$3^{a} \pm 0.71$
	0.3%	++	$14^{bc} \pm 0.41$	$6^{bc} \pm 1.08$
Activated charcoal	150	+++	$23^{g} \pm 0.82$	$3^{a} \pm 0.41$
	250	+++	$18^{ef} \pm 0.82$	$4^{ab} \pm 0.71$
	350	++	$16^{cde} \pm 0.41$	$7^{c} \pm 1.08$

to the culture medium reduced phenolic browning and prolonged survival and frequent sub-culturing was also practiced to overcome the effects of phenolic compounds (McGranahan *et al.*, 1). McGranahan *et al.* (1) also suggested that frequent sub-culturing of the explants on to fresh medium improved the quality of cultures and increased the survival rate.

ACKNOWLEDGMENTS

The authors are grateful to Indian Council of Agricultural Research, New Delhi, India, for providing financial assistance.

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Received : November, 2014; Revised : February, 2017; Accepted : April, 2017