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Research Article

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Effect of different growth regulator combinations on *in vitro* callusing of walnut (*J. regia* L.)

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Summary

The present investigation on effect of different growth regulator combinations on *in vitro* callusing of walnut (*J. regia* L.) studies using MS medium was carried out in order to document the available genetic variability in walnut germplasm and to select elite walnut genotypes possessing superior attributes and quality traits. During the survey, data was recorded on one hundred fifty two (152) walnut trees growing in different areas of Kashmir valley. The study also involved establishment of response of elite walnut selections to different plant growth regulators in shoot morphogenesis. Woody species have been found to be far more difficult to clone *in vitro* than herbaceous plants. Poor response of the explants from mature woody species to *in vitro* manipulation is usually associated with the problem of browning and explant necrosis. The present studies were conducted on forced explants from three walnut selections (SKUAST 002, SKUAST 008, SKUAST 010). Murashiage and Skoog's basal medium supplemented with 0.3 mg/l^{-1} Benzylamino purine and 0.1 mg/l^{-1} indole-3-butyric acid the success varied between zero to 63.88 per cent. Data recorded in per cent was transformed to arc sin^{-1} values. Accessions revealed a non-significant difference in influencing the callusing of explant tissues after application of the growth regulators. The values for SKUA-002, SKUA-008 and SKUA-010 were 28.08, 25.25 and 28.52 corresponding 27.91, 24.68 and 28.55 per cent callusing, respectively.

Key words : Walnut, *Juglans regia* L., Variability, Shoot morphogenetic response, Callusing of walnut (*J. regia* L.)

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Introduction

The persian walnut (*Juglans regia* L.), known as the English walnut, belongs to the family Juglandaceae. English walnut has its origin in the eastern Europe, Asia minor and points eastward to Himalayan mountains. The native habitat of walnut extends from the Carpathian mountains to Europe across Turkey, Iraq, Afghanistan, South Russia and further eastward into the foot hills of the Himalayas. In India walnuts are usually grown in the mid hill areas of Jammu and Kashmir, Himachal Pradesh, and upper hills of Uttarakhand and Arunachal Pradesh. The soil most suitable for its cultivation should be well-drained and deep silt loamy containing organic matter in abundance. It should not have a fluctuating water level, hard pan and/or sandy sub-soil with alkaline reaction. A soil 2.5 to 3.0 m deep gives best results because the roots can penetrate deep and utilize residual soil moisture

during dry spell and also make available sufficient nutrients. Furthermore, availability of sufficient moisture in the leaves can reduce the damage due to sun burning of leaves, shoots and young fruits. Walnut is grown commercially in about 48 countries with an area of 66, 58, 966 hectares. The world walnut production is about 16, 70, 109 MT. The chief walnut producing countries are China (22%), USA (20%), Iran (12%) and Turkey (10%) (Anonymous, 2007 and 1984). India accounts for about 2.0 per cent of the world production. In India, Jammu and Kashmir is leading both in area as well as in production with an area of 82.04 thousand ha and production of 146.78 thousand tonnes. However, the productivity level of 1.79 t ha^{-1} is far below than other countries. Himachal Pradesh has an area of 6.54 thousand ha with a production of 1.24 thousand tonnes and productivity level of 0.19 t ha^{-1}; while Uttarakhand has an area of 19.26 thousand ha with a production of 8.73 thousand tonnes and productivity level of 0.45 t/ha and Arunachal Pradesh has an area of 2285 ha with a production of about 51 tonnes and productivity level of 0.022 t/ha.In the state of Jammu and Kashmir, Anantnag is the leading district both in area as well as production corresponding to an area of 13647 ha and production of 41180 tonnes with a productivity level of 3.01 t ha^{-1}, followed by the Kupwara district that covers an area of 8175 ha with 22103 tonnes production and a productivity level of a 2.70 t ha^{-1}. Kulgam ranks 6^{th} in area and 3^{rd} in production in the J&K state and has the highest productivity of 3.52 t ha^{-1}, which is even higher than that of USA. This indicates that the state has the right type of agro-climatic conditions and vast potential to produce export quality walnut and kernels. Micro propagation studies in walnut are not so well established nor any fool proof protocol is yet developed for efficient and faster multiplication of superior plants. The presence of phenolic compounds and entophytic bacteria are still the main limiting factors for establishing plant micro propagation in walnuts. The use of young vegetal material is the usual technique for *in vitro* set up of walnut (Driver and Kuniyuki, 1984 and Jay-Allemand *et al.*, 1993). Quality in regeneration of *in vitro* plant material is correlated with maintenance of mother plants in the controlled environments, with regular hormone application and proper choice of physiological stage for collecting materials. The correct temperature in growth chambers is essential for a proper regeneration as well for subsequent multiplication (Dolcet-Sanjuan *et al.*, 1993). The addition of PVP to the culture medium as well as the substitution of agar by gelrite are the main factors reported for the control of phenolic compounds.

The current methodology of woody crop rooting by a bietapic process is well documented in walnut (Driver *et al.*, 1984) with the use of IBA. Walnut is hard to propagate through micro propagation. Various attempts have been made using different types of explants, media, culture condition and rooting techniques (Driver and Kuniyuki, 1984). Poor proliferation and rooting rate is one of the main obstacles that limit the micro propagation efficiency in walnut. Intensive and well planned research is needed to develop a perfect protocol for micro propagation for this crop. Genotype plays a major role in vegetative propagation, in particular for micro propagation.

In many cases the propagation ratio can be improved by using a stronger cytokinin or increasing its concentration. However, this can sometimes have detrimental effects in the later stages of micro propagation. Micro propagation studies have also been carried out in some other species of nuts and similar trees like hazelnut (Radojevic *et al.*, 1975; Mele and Messeguer, 1983 and Perez *et al.*, 1983), chestnut (Vieter and Vieiter, 1980) and almond (Mehra and Mehra, 1974). But reports on *in vitro* walnut culture are scarce.

Resource and Research Methods

Shoot morphogenetic studies :

The investigation was carried out at the Tissue Culture Laboratory of Regional Research Station Wadura, SKUAST (K), during the year 2014-2015.

The investigation involved shoot morphogenetic response in walnut to different treatment combinations of growth hormones.

Plant materials used :

The mature and bearing elite genotypes of walnut selections *viz.*, SKUAST-002, SKUAST-008 and SKUAST-010 growing at the Experimental Farm of the Division of Polmology at the main campus Shalimar were used as stock plants in the present study. The plant materials were subjected to uniform cultural practices.

Chemicals used :

The major and minor elements required for the preparation of media were obtained from the Hi Media Pvt. Ltd. The amino acids, vitamins and plant growth

regulators used were obtained from the Sigma chemicals.

Glass wares :

The glass ware used for the experiment was obtained from the Borosil Glass-ware. Before use the glassware was soaked in potassium dichromate- nitric acid solution for six hours followed by thorough washing in a jet of tap water so as to completely remove all traces of dichromate solution. They were then soaked in detergent solution (Teepol 1%) overnight and were thoroughly washed in tap water and rinsed twice with double distilled water. The glass-ware was then dried in hot air oven at 100°C for 24 h and later stored under aseptic conditions till use.

Culture media :

Selection of culture media :

Murashige and Skoog (1962) medium was used for the present investigation.

Inositol (100mg), sucrose (30g) and agar (7.0g) required for one litre culture medium were added directly at the time of preparation of culture media.

Adjutants to the basal medium :

The MS basal medium was appropriately supplemented with vitamins, aminoacids and growth regulators. Stock solution of organics (vitamins and amino acids) were prepared in double distilled water.

Preparation of the culture media :

The required quantity of sucrose was dissolved in double distilled water and to this stock solution macronutrients, micronutrients, vitamins and growth substance were added as per treatment required. The media of various formulations were prepared.

The pH of the medium was adjusted to 5.5 with HCl or 0.1 N NaOH prior to the addition of agar. Agar (7.0 g^{-l}) was dissolved in boiling distilled water and added to the medium and the volume was made up with distilled water. The medium was boiled and the hot medium was then immediately dispensed in the test tubes or conical flasks which were plugged tightly with non-absorbent cotton plugs and covered with aluminum foil. The medium was autoclaved at 15 psi (121°C) for 15 minutes (Dodds and Robert, 1982).

Transfer area and aseptic manipulations :

All the aseptic manipulations like surface sterilization, preparation and inoculation of explants and subsequent subculturing were carried out under hood of clean laminar air flow chamber. The working table of the laminar air flow chamber was first surface sterilized with absolute alcohol followed by ultra violet light for 30 minutes. The forceps and scalpel used for the inoculation

Table A : Composition and preparation of stock solutions for Murashige and Skoog (1962) medium

Stock solution	Components	Chemical formula	Quantity (mg l^{-1})	Quantity for 20 litres	Quantity of stock solution prepared	Conc. of stock solution
A	Ammonium nitrate	NH_4NO_3	1650	33.00 g	200 ml	100 x
B	Potassium nitrate	KNO_3	1900	38.00 g	200 ml	100 x
C	Potassium iodide	KI	0.83	16.6 mg		
	Boric acid	H_3BO_3	6.2	124 mg		
	Potassium phosphate, monobasic	KH_2PO_4	170	3.4 g	200 ml	100 x
	Molybdic acid (Sodium salt) $2H_2O$	$Na_2M_0O_4.2\ H_2O$	0.25	5 mg		
	Cobalt chloride hexahydrate	$CoCl_2.\ 6H_2O$	0.025	0.5 mg		
D	Calcium chloride, dihydrate	$CaCl_2.\ 2H_2O$	440	8.8 g	200 ml	100 x
E	Magnesium sulphate, heptahydrate	$MgSO_4.\ 7H_2O$	370	7.4 g		
	Zinc sulphate, heptahydrate	$ZnSO_4.\ 7H_2O$	8.6	172 mg	200 ml	100 x
	Managanese sulfatem tetrahydrate	$MnSO_4.\ 4H_2O$	22.3	446 mg		
	Cupric sulfate pentahydrate	$CuSO_4.\ 5H_2O$	0.025	0.5 mg		
F	Ethylenediaminetera acetic acid disodium salt	$Na_2EDTA.2H_2O$	37.3	746	200 ml	100 x
	Ferrous sulfate heptahydrate	$FeSO_4.7H_2O$	27.8	556 mg		
G	Glycine	-	2.00	40 mg		
	Nicotinic acid	-	0.5	10	200 ml	100 x
	Pyridoxine HCl	-	0.5	10		
	Thiamine HCl	-	0.1	2		

as well as the Petri dishes were first steam sterilized in an autoclave at 121^{0}C for 20 minutes and later flame sterilized before each inoculation. The hands were cleaned and wiped with alcohol before working. Further, the standard general procedure for sterile technique suggested by Street (1977) was followed.

Incubation chamber :

The cultures were incubated at 24±1^{o}C in an air condition culture room with a 16 h photoperiod (3500 lux).

Collection and preparation of explant :

Forcing of stock plant:

This experiment consisted of field grown adult shoots and artificially forced walnut cuttings of the genotypes SKUAST-002, SKUAST-008 and SKUAST-010 of walnut. For forcing the dormant cuttings of 15-20 cm length were collected in January. Dormant cuttings were treated with 0.2 per cent captan and stored in cold store at 4±3^{o}C in polythene bags for two months.Cuttings were withdrawn from cold store as and when required and placed in jars containing distilled water and kept in incubation chamber. The cuttings were incubated at 24±1^{o}C in an air condition room with 16 hours of Photoperiod (3.5 lux). Sprouting of dormant buds occurred after one month of transferring to incubation chamber which served as explant source. Shoot tips from all the three genotypes were excised and cultured on semi-solid and stationery liquid medium for establishment. Each treatment combination consisted of 25 explants and was replicated 3 times for each genotype.

Surface sterilization and inoculation :

The explants were surface sterilized with 0.1 per cent (w/v) mercuric chloride solution for 10 minutes and then washed thoroughly with double distilled sterile water (5-6 times) under aseptic conditions in the laminar flow chamber. The explants were then placed on the medium in such a manner that conformed to the original polarity and were exposed above the surface of medium.

Details of *in vitro* studies:

Details of growth regulators:

The explants were cultured in Murashige and Skoogs (1962) basal medium (MS) supplemented with different concentrations of growth regulators. The details of the combinations of different concentration of growth regulators are presented Table B.

Table B : Details of growth regulators

Growth regulators	Concentration used			
BAP	0.3 mg l^{-1}	0.6 mg l^{-1}	0.9 mg l^{-1}	1.2 mg l^{-1}
IBA	0.1 mg l^{-1}	-	-	-

Treatment combinations used

T_1 = BAP 0.3mg l^{-1} + IBA 0.1mg l^{-1}
T_2 = BAP 0.6 mg l^{-1} + IBA 0.1 mg l^{-1}
T_3 = BAP 0.9 mg l^{-1} + IBA 0.1 mg l^{-1}
T_4 = BAP 1.2 mg l^{-1} + IBA 0.1 mg l^{-1}

The data recorded on various parameters of tissue culture studies were put to statistical analyses. Values in per cent were transformed to arc sin (angular) or square root transformation before analysis. The mean performance of the cultivars and growth regulator combinations was presented for experimental findings alongwith their interaction (cultivars x growth regulator combination).

Survival of shoot apices :

Survival of explants was recorded within 8 days of inoculation in each treatment combination for each cultivar and expressed as per cent as follows :

$$\text{Per cent survival of explants} = \frac{\text{Number of explants survived}}{\text{Total number of explants culture}} \times 100$$

Per cent growing cultures :

Number of explants that showed growth were also recorded within 3 weeks of inoculation and expressed in per cent.

$$\text{Per cent growing cultures} = \frac{\text{Number of explants showing growth}}{\text{Total number of explants cultured}} \times 100$$

Explants showing browning :

Explants that showed browning within 8 days were recorded and expressed as per cent for each treatment.

$$\text{Per cent explant browning} = \frac{\text{Number of explants browned}}{\text{Total number of explants cultured}} \times 100$$

Mean browning score per explant :

Explants that showed browning were scored for browning intensity as under :

0 = No browning
1 = Browning on excised wound tissue.
2 = Partial browning on outer tissue of explant
3 = Complete browning of the outer tissue of explant
4 = Browning of the entire explant tissue.

Explants showing substrates browning :

Substrate browning (irrespective of explant browning) was recorded for each culture treatment in a genotype and expressed as per cent.

$$\text{Per cent substrate browning} = \frac{\text{Number of culture tubes showing media browning}}{\text{Total number of explants cultured}} \times 100$$

Volume of browned media per culture :

Volume of the browned media was calculated on the basis of diameter of browned media surrounding the explant and depth of the browned media by the following relationship.

$$V = ® xr \times d$$

where,

V = Volume in cubic millimetres.

r = Radius of the browned media in millimetres

d = Depth of the browned media in millimetres.

Browning score of the explant and volume of browning media in each treatment was worked out per explant basis. The values were expressed finally as volume of browned media $(mm)^3$ x 1/100 per explant.

Observation on growth rating of explant and percentage of callused cultures was recorded after 30 days of culture initiation and growing culture were again transferred to fresh medium of the same composition. were again transferred to fresh medium of the same composition.

Growth rating of explants :

Explants that revived and showed growth were scored for growth vigour as under :

1 = Low growth rate

2 = Medium growth rate

3 = High growth rate.

Percentage of callused cultures :

Explants that survived and revealed growth were scored for callusing and expressed as per cent.

$$\text{Per cent cultivars showing callusing} = \frac{\text{Number of explants expressing callus}}{\text{Number of surviving and growing explants}} \times 100$$

Statistical analysis of the data :

The data recorded for phenotypic variability of trait were put to statistical analyses and different parameters estimated.

Table C : Analysis of variance

Source of variation	Df	Expected mean	Sum of squares	Variance ratio
Replications (years)	y^{-1}	MS_1	$\sigma^2e+g\sigma^2y$	MS_1/MS_3
Cultivars (genotypes)	g^{-1}	MS_2	$\sigma^2e+y\sigma^2g$	MS_2/MS_3
Replications x cultivars	(y^{-1}) (g^{-1})	MS_3	σ^2e	

Genetic variability studies :

Data for each trait of each earmarked *in-situ* tree was recorded over two crop seasons (*Kharif* 2008 and *Kharif* 2009) and statically analyzed.

Estimation of variability parameters :

Different variability parameters estimated were as under.

Mean and range :

The population mean and range of each trait was expressed in the same units as that of the trait.

Phenotypic co-efficient of variation

Phenotypic co-efficient of variation (PCV) was expressed as:

$$PCV = \frac{\sqrt{\sigma^2 p}}{\bar{X}} \times 100$$

Genotypic co-efficient of variation :

Genotypic co-efficient of variation (GCV) was expressed as:

$$GCV = \frac{\sqrt{\sigma^2 g}}{\bar{X}} \times 100$$

Components of variance :

The variance for each trait comprised variance arising from the cultivars difference (genotypic variance) and that arising from the environmental factor (in this case years). The expected mean sum of squares would be as under:

$$\text{MS for cultivars} = \pm^2e + y \pm^2g = MS_2$$

$$\text{MS for error} = \pm^2e = MS_3$$

$$\text{Genotyic variance}, \sigma^2 g = \frac{Ms_2 - Ms_3}{Y}$$

$$\text{Phenotypic variance} = \pm^2P = \pm^2g +\text{-} \pm^2e$$

Heritability (broad sense) :

It was measured as the ratio of genotypic variance

to phenotypic variance.

$h^2(bs) = \pm^2 g / \pm^2 p$

Genetic gain :

Genetic gain (% of mean) was expressed as :

$$\text{Genetic gain} = \frac{h^2(b.s) \times \sigma p \times k}{\overline{X}}$$

where,

$h^2(b.s)$ = Heritability in broad sense,

$\pm p$ = Phenotypic standard deviation

K = Selection intensity (the value of k at 5% was taken as 2.06) and

$\overline{X}$ = Population mean of the trait.

Research Findings and Discussion

Explants of three elite walnut cultivars *viz.*, SKUA-002, SKUA-008 and SKUA-010 were studied for their response to shoot morphogenesis under micro propagation (tissue culture) techniques. The standard basal medium of Murashige and Skoog (1962) was supplemented with BAP (0.3, 0.6, 0.9 and 1.2 mg l^{-1}) together with a uniform concentration of 0.1 mg l^{-1} of IBA. The results pertaining to different parameters of *in vitro* studies are briefly presented in the following.s Mean callusing of the explants from different walnut accessions after the application of different growth regulator combination (Table 1) revealed that in the explants, that survived, the success varied between zero to 63.88 per cent. Data recorded in per cent was transformed to arc sin^{-1} values. Accessions revealed a non-significant difference in influencing the callusing of explants tissues after application of the growth regulators.

Values for SKUA-002, SKUA-008 and SKUA-010 were 28.08, 25.25 and 28.52 corresponding 27.91, 24.68 and 28.55 per cent callusing, respectively (Table 1).

Among the growth regulator combinations there was significant increase in the callusing of the explant tissues as the concentration of BAP increased at each individual level of this concentration. The transformed mean values of 4.055, 17.706, 35.660 and 57.722 corresponding to per cent callusing of 0.00, 12.77, 34.13 and 61.90 in respect of T_1 (BAP 0.3 mg l^{-1} + IBA 0.1 mg l^{-1}), T_2 (BAP 0.6 mg l^{-1} + IBA 0.1 mg l^{-1}), T_3 (BAP 0.9 mg l^{-1} + IBA 0.1 mg l^{-1}) and T_4 (BAP 1.2 mg l^{-1} +IBA 0.1 mg l^{-1}) were all significantly different from each other.

The interaction effect on the callusing due to accessions x growth regulators revealed that none of the accessions had significant difference at individual growth regulator combination. However, in each accessions significantly higher callusing was recorded as the concentration of BAP increased from 0.3 mgl^{-1} to 1.2 mgl^{-1} together with a constant application 0.1mg l^{-1} IBA except in the case of SKUA-008 between T_1 (BAP 0.3 mg l^{-1} + IBA 0.1 mg l^{-1}) and T_2 (BAP 0.6 mg l^{-1} + IBA 0.1 mg l^{-1}).

Formation of callus in the explants showing growth ranged between zero to 63.88 per cent. Accessions as such had no impact on the callusing of explants. Increase in the concentration of BAP (from 0.3 to 1.2 mg l^{-1}) along with IBA (0.1mg l^{-1}) significantly increased the callusing behaviour of the growing explants. The increase was from zero at 0.3 mg l^{-1} BAP concentration to 12.77, 34.13 and 61.29 per cent at 0.6,0.9 and 1.2 mg l^{-1} of BAP, respectively. Interaction effects were also significant in most of the cases.

Table 1 : Effect of different growth regulator combinations on *in vitro* callusing of walnut (*J. regia* L.)

Walnut accessions	Growth regulators				Mean
	T_1	T_2	T_3	T_4	
SKUA-002	4.055* (0.000)	20.201 (15.000)	36.154 (35.000)	51.923 (61.666)	28.085 (27.910)
SKUA-008	4.055 (0.000)	12.703 (8.333)	34.239 (32.060)	49.998 (58.333)	25.249 (24.680)
SKUA-010	4.055 (0.000)	20.207 (15.000)	36.585 (35.333)	53.244 (63.880)	28.523 (28.55)
Mean	4.055 (0.000)	17.706 (12.770)	35.660 (34.130)	57.722 (61.290)	27.285 (27.060)

Table 2 : Values in parentheses are the arc sin transformation values of the original data

Critical difference at 5%	
Accessions (AC)	7.662
Growth regulators (GR)	8.847
Interaction (AC x GR)	15.324

T_1 = BAP 0.3 mg l^{-1} + IBA 0.1 mg l^{-1}, T_2 = BAP 0.6 mg l^{-1} + IBA 0.1 mg l^{-1},
T_3 = BAP 0.9 mg l^{-1} + IBA 0.1 mg l^{-1}, T_4 = BAP 1.2 mg l^{-1} + IBA 0.1 mg l^{-1}

Somers *et al*. (1982) reported that callus formation has been frequently found when embryogenic explants were cultured that also revealed other morphogenetic response. Mature embryos cultured on MS or WP media revealed increase in the callusing as the concentration of BAP was increased.Cheema and Mehra (1982) observed callus formation in seed endosperm (mature and immature) on MS medium containing IBA (2.0 mg l^{-1}) and NAA (0.2 mg l^{-1}). The cultures developed roots and shoots when the media was also supplemented with cytokinins such as Kn (2 mg l^{-1}). Rodriguez *et al.* (1983) studied *in vitro* propagation of 5 to 7 week old plants. Callus formation preceded root or multiple shoot bud induction. The morphology varied in accordance with the auxins present in the medium of culture. Callus induction in miniature cotyledons when cultured on K (h) medium supplemented with 2,4-D (0.1-10 mg l^{-1}) and Kn (0.1-1 mg l^{-1}) was rapid. The callus was nodular and compact with a tendency of browning which increased gradually as the 2,4-D concentration increased and the period of explant culture was prolonged. No organ induction was possible from the callus formed in the presence of this growth regulator. However, callus induction on the same media but supplemented with NAA (0.1-10 mg l^{-1}) or IBA (0.1 mg l^{-1}) was rapid and more morphogenetic. The callus obtained was fragile, greenish, with less tendency to browning, of more rapid growth and produced a greater number of anthocyanins especially in presence of NAA (100 mg l^{-1}) and Kn (0.5 mg l^{-1}). Greater root initiation was also observed on this medium.

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