International Journal of
Plant Biology

MDPI

Article

In Vitro Prevention of Browning in Persian Walnut (*Juglans regia* L.) cv. Sulaiman

Suhail Nazir Bhat [1,*], Aroosa Khalil [1], Nowsheen Nazir [1], Mohammad Amin Mir [1], Imran Khan [2], Syed Shoaib Mubashir [3], Mohammad Saleem Dar [4], Shabir Hussain Wani [5,*] and Mohammad Anwar Hossain [6,*]

1 Division of Fruit Science, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar-Srinagar (J and K), Srinagar 190001, India
2 Division of Agricultural Statistics, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar-Srinagar (J and K), Srinagar 190001, India
3 Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar-Srinagar (J and K), Srinagar 190001, India
4 Mountain Research Centre for Field Crops, SKUAST-Kashmir (J and K), Kulgam 192101, India
5 Mountain Research Center for Field Crops Khudwani, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar 192101, India
6 Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
* Correspondence: suhailnb99@gmail.com (S.N.B.); shabirhussainwani@gmail.com (S.H.W.); anwargpb@bau.edu.bd (M.A.H.)

Abstract: The present investigation was undertaken to standardize the media and the anti-browning regime in order to minimize the phenolic browning of an in vitro culture of Persian walnut cv. Sulaiman. The experiments involved two types of explants, forced and unforced shoot tips, two types of media, Driver and Kuniyuki Walnut (DKW) medium andMurashige and Skoogs (MS) medium, and three types of anti-browning agents, namely, Polyvinylpyrrolidone, ascorbic acid and activated charcoal at 150, 350 and 550 mg/L each. The investigation was replicated thrice under a completely randomized design. Forced shoot tips of cv. Sulaiman on DKW medium showed the best performance in terms of least browning (13.6 ± 10.5%) and highest survival percentage of explants (74.5 ± 2.4%) when treated with ascorbic acid at 550 mg/L. However, unforced shoot tips in MS medium did not perform well and manifested maximum browning (52.9 ± 5.2%). Based on the results, we conclude that incorporation of ascorbic acid in the DKW medium significantly reduced the media and explant browning, thus, it could set the basis of successful in vitro-propagation of walnuts.

Keywords: explant; media; in vitro culture; browning; Persian walnut

check for updates

Citation: Bhat, S.N.; Khalil, A.; Nazir, N.; Mir, M.A.; Khan, I.; Mubashir, S.S.; Dar, M.S.; Wani, S.H.; Hossain, M.A. In Vitro Prevention of Browning in Persian Walnut (*Juglans regia* L.) cv. Sulaiman. *Int. J. Plant Biol.* **2022**, *13*, 330–342. https://doi.org/10.3390/ijpb13030027

Academic Editor: Adriano Sofo

Received: 11 May 2022
Accepted: 20 August 2022
Published: 25 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The Persian walnut (*Juglans regia* L., family: juglandaceae) is one of the important species of the genus Juglans. It originated in Central Asia and its wild and semi-cultivated ancestors have been found in the Balkans across Turkey, China and Eastern Himalaya [1–3]. Later, it spread to Western Asia and Eastern Europe from Xinjiang province of China, parts of Kazakistan, Uzbakistan, Kyrgistan, and from Iran and the Central Himalayan region [4,5].

The walnut tree is a prominent temperate nut crop having great potential for the production of nuts and is rich in nutrients and mineral matter [6]. Nuts are abundant in proteins, fats, mineral elements and are a consolidated source of energy [7]. Being among the high ranked nut fruit crops, the world's annual production is 4.409 million tonnes out of an area around 1.305 million hactares [8]. Walnut plantations are generally derived from seedling origins (high heterozygosis and long gestation periods) which is undesirable [9]. Although it contributes to the large walnut gene pool, due to poor

performance through vegetative propagation (grafting, budding and cutting) under natural conditions, multiplication of elite genotypes to a desired extent becomes unattainable [10].

Micropropagation has revolutionized the business of commercial nurseries in the last two decades. The Persian walnut is the most commonly cultivated and horticulturally established tree species for nut yield [11,12]. The potential of micropropagation of walnuts was initiated from explants, ranging from embryos to apical buds or petioles, having been extensively exploited a few decades back, since the first study on stem culture of black walnuts (*J. nigra*) [13]. Several authors have developed in vitro propagation protocols for various genotypes. However, recalcitrance of walnuts towards tissue culture have made it difficult for the micropropagation of newly developed genotypes [3,14–19]. Moreover, genotype dependence, as well as the low reproducibility of these protocols, have made it's in vitro propagation more complicated. Most of these experiments focused on the browning phenomenon, elongation of shoots or buds, induction of roots, germination of embryos or somatic embryogenesis [6,20,21].

Rahman [22] studied the DKW medium and MS medium in which he found that DKW clearly showed dominating performance over MS medium in various tissue culture stages, such as callus mediated shooting and multiple shoot induction in Clitoriaternatea and Baptisia australis. Yegizbayevaet al. [23] also reported the best results on corrected DKW formulations for proliferation, rooting or ex vitro survival of walnut genotypes. Additionally, various techniques for inhibition of browning in microcuttings of English walnut have been examined, in which different media (DKW, DKW and MS) and anti-browning agents (activated charcoal and ascorbic acid) were employed for initiation of two cultivars, Chandler and Jamal. Activated charcoal supplemented medium of DKW was found to be the optimal medium, compared to the rest of the combinations [24].

Xiao-Dong et al. [25] focused on in vitro induction of the high-quality callus of *Juglans regia* L.cv, 'Xiangling', using different growth regulator levels in DKW basal medium. In their study, a DKW medium consisting of 1 mg/L BA, 2 mg/L KT, 250 mg/L glutamine, 500 mg/L casein hydrolysate, 200 mg/L Vc, 50 g/L sucrose and 2.8 g/L Gelrite was the best culture medium for induction of callus in immature cotyledons and embryos. Rout et al. [26] observed that browning of the medium resulted due to the exudation of polyphenolic compounds from the sliced end of rose explants, which could be suppressed by the addition of some important compounds, such as polyvinyl pyrolidone (PVP), citric acid, ascorbic acid, activated charcoal, and glutamine, or by frequently subculturing.

In vitro oxidative or phenolic browning of explants and media, in addition to other fungal and bacterial contamination, has become a major recalcitrant problem in the explant regeneration of walnut plants [27]. Different researchers have employed various anti-oxidant agents, viz., polyvinyl pyrolidone (PVP), activated charcoal and ascorbic acid, at varying concentrations to reduce the browning of media and tissues and have got varying results [28–31]. Keeping the above-mentioned constraints in mind, the aim of our research was to standardize the optimal in vitro medium and anti-oxidant regime for minimizing explant and medium browning.

2. Materials and Methods

The experiments were performed at the Commercial Tissue Culture Unit of Seven Star Fruits Pvt. Ltd., Aurangabad (MH) and the Tissue Culture Laboratory of Fruit Science Division, SKUAST-Kashmir, Shalimar, India, over the period 2019–2020. The investigation (Table 1) involved the study of different types of anti-browning treatments to explants and two media formulations to check in vitro browning of forced (treatments given to stock material to induce shooting under controlled laboratory conditions) and unforced shoot tips of "Sulaiman", a promising variety of walnut (*J. regia* L.). Actively growing unforced shoot tip explants 2–3 cm long were excised from mature walnut bearing trees of the Sulaiman variety, grown in the experimental walnutblock at the main campus of SKUAST-K, Shalimar, in the month of June-July 2019. Shoot tips were collected in flasks

containing tap water, brought to the laboratory and washed thoroughly and kept under gushing tap water for 1 h.

Table 1. Salient features of the anti-browning experiment.

Factors and Size of Factorial Experiment [a]	Levels of Factors [b]	Parameters [c]	Design of Experiment [d]
E. Explant source (2)	E1-Unforced shoot tip E2-Forced shoot tip	1–4	CRD
M. Media (2)	M1-MS M2-DKW		
A. Anti-browning agents (3)	A1-Polyvinylpyrrolidone A2-Ascorbic acid A3-Activated charcoal		
C. Concentrations (mg/L) (3)	C1-150 C2-350 C3-550		

[a] = Figures in the parentheses refer to the number of levels of each factor; [b] = A1-Polyvinylpyrrolidone, A2-Ascorbic acid, A3-Activated charcoal; MS, Murashige and Skoog's (1962) medium, DKW, Driver and Kuniyuki Walnut (1984) medium; [c] = 1. Explants showing browning (%); 2. Explants showing media browning (%); 3. Mean browning score per explant (0–4); 4. Explant survival (%); [d] = CRD (completelyrandomizedblockdesign). Each treatment combination was replicated thrice and assigned 24 experimental units each.

For obtaining forced explants, pruned stock bud wood was collected from experimental field stock plants of the Sulaiman variety of walnut during the dormant months of November-December. In order to force (Figure 1) the latent buds in the dormant wood (sub terminal or terminal potion, 15–20 cm long and 10–15 mm in diameter) were taken from bearing walnut trees. The dormant wood was first treated with Captan (@ 0.2 per cent), followed by cold storage at 4 ± 3 °C in poly-bags till further use. Forcing was done as per the procedure elucidated by [32].



(**a**) (**b**)

Figure 1. *Cont.*



(**c**)

Figure 1. Various stages of forcing of dormant walnut cuttings in the growth chamber: (**a**) 0 day after incubation; (**b**) 10 days after incubation; (**c**) Complete leafing out after 20 days of incubation.

The explant sterilization was done in 0.1% $HgCl_2$ for 5 min, which was followed by 70% ethanol treatment for 10s. After sterilization, the explants were washed 3 to 5 times under a laminar hood in sterilized water. Explants were inoculated on the medium by taking polarity into consideration and the buds were kept exposed. The culture tubes were then incubated at 25 ± 2 °C in a growth chamber with a 16/8 h photoperiod under light intensity of 3500 lux. Explants were shifted to fresh media after every 2–3 days of inoculation. Observations with regard to per cent explant survival (explants which showed signs of growth) and browned culture percentage were recorded within 3 ± 1 weeks of inoculation.

Two different types of the media i.e., MS [33] and DKW [34] media, were used in the present investigation. The composition of the different media utilized in the experiments, and details regarding stock solution preparations, are furnished in Table 2. The required quantities of sucrose, stock solutions of macronutrients, micronutrients, vitamins and anti-browning agents were dissolved in double distilled water as per treatment requirements [35]. The medium was adjusted to a pH of 5.8 with 0.1 N hydrochloric acid or 0.1 N sodium hydoxide, before adding agar to it. A gelling agent (Agar −7 g/L or Gelrite-2.8 g/L) was dissolved in boiled distilled water before adding it to the media and double distilled water was used to make the final volume. The hot media was poured into the test tubes and flasks just after boiling and, after tight plugging and wrapping with Al foil, the media were then autoclaved at 15 psi (121 °C for 15 min) [36].

Experimental Design and Statistical Data Analysis

The data generated from the experiments were subjected to statistical analysis (Analysis of variance) by using IBM-SPSS v.26.0,Chicago, IL, Statistical Software tool. The experimental model applied was of a completely randomized design. Treatments were replicated 3 times comprised of 24 explants per replication. Each container was the basic experimental unit; therefore, the average of all the containers was used as individual data for analysis.

Table 2. Salt composition (mg/L) of each culture medium studied.

	MS	DKW
NH_4NO_3	1650	1416
Ca $(NO_3)_2.4H_2O$	-	1968
KNO_3	1900	-
$MgSO_47H_2O$	370	740
KH_2PO_4	170	265
K_2SO_4	-	1559
$CaCl_22H_2O$	440	149
H_3 BO_3	6.2	4.8
KI	0.83	-
$Na_2MoO_42H_2O$	0.25	0.39
$CoCl_26$ H_2O	0.025	-
$CuSO_4$ $5H_2O$	0.025	0.25
$ZnSO_4$ $7H_2O$	8.6	-
$MnSO_4$ $4H_2O$	22.3	33.5
Na_2 EDTA $2H_2O$	37.3	45.4
$FeSO_47H_2O$	27.8	33.8
Glycine	2	2
Nicotinic acid	0.5	1
Thiamine HCl	0.1	2
Pyridoxine HCl	0.5	-
Myo-inositol	100	100

3. Results

In the present studies in vitro browning was measured both on explants and nutrient media under the following headings:

3.1. Medium, Anti-Browning Regime and Explant Browning

The interaction effect of anti-browning agents, concentration and source of explant on percentage of browned explants was found to be significant. The minimum percentage of browned explants (13.6 ± 10.5 per cent) was recorded in forced shoot tips treated with ascorbic acid at 550 mg/L, while, the maximum explant browning (52.9 ± 5.2 per cent) was obtained in unforced shoot tips treated with Polyvinylpyrrolidone (PVP) at 150 mg/L (Table 3).

Table 3. Influence of anti-browning regime and explant source on explant browning (%) of walnut (*Juglans regia* L.) var. Sulaiman.

		Explant (E)					
		E1			E2		
Concentration (C)		C1	C2	C3	C1	C2	C3
Anti browng Agent (A)	A1	52.9 ± 5.2	33.8 ± 5.3	31.4 ± 9.0	44.2 ± 11.0	22.2 ± 5.1	21.2 ± 7.0
	A2	39.5 ± 5.4	32.7 ± 6.3	30.7 ± 4.9	35.2 ± 9.8	15.0 ± 6.1	13.6 ± 10.5
	A3	49.0 ± 5.1	33.8 ± 7.7	31.8 ± 4.2	42.1 ± 6.0	24.2 ± 6.1	22.3 ± 7.9

CD $_{(0.05)}$: E*C*A = 1.47. A1—Polyvinylpyrrolidone; A2—Ascorbic acid; A3—Activated charcoal; E1-Un-forced shoot tip; E2—Forced shoot tip; C1, 150 mg/L; C2, 350 mg/L; C3, 550 mg/L. The treatments were replicated thrice containing 24 explants each. The values in the table represent mean ± SD of the treatment combinations. Data was subjected to ANOVA (see Table A1). F-Test ($p \leq 0.05$) and R Squared = 0.932 (Adjusted R Squared = 0.898) were also performed.

3.2. Medium, Anti-Browning Regime and Medium Browning

Table 4 shows how media and explant affected media browning percentage significantly. The highest percentage of media browning (44.6 per cent) was recorded in unforced shoot tips in MS medium, while the lowest value (26.4 per cent) was observed in forced shoot tips in DKW medium.Furthermore, the interaction effect between concentration and

anti-browning agent on media browning percentage was found to be significant. Maximum media browning (58.4 ± 4.6 per cent) was recorded in PVP at 150 mg/L, while the minimum value (21.3 ± 1.3 per cent) was observed in ascorbic acid at 550 mg/L.

Table 4. Influence of anti-browning regime, explant source and media on media browning (%) of walnut (*Juglans regia* L.) var. Sulaiman.

		Explant (E)					
		E1			E2		
Media (M)	**Concentration (C)**	C1	C2	C3	C1	C2	C3
M1	A1	58.4 ± 4.6	44.6 ± 2.0	41.7 ± 0.7	52.5 ± 2.0	31.3 ± 0.3	27.8 ± 0.8
	A2	49.1 ± 1.7	39.2 ± 2.1	33.3 ± 1.4	40.5 ± 0.7	25.4 ± 1.1	22.7 ± 1.2
	A3	57.9 ± 2.5	41.7 ± 1.0	36.0 ± 1.3	47.8 ± 3.4	28.6 ± 2.0	26.9 ± 1.3
M2	A1	55.5 ± 4.1	44.2 ± 1.8	39.2 ± 0.6	50.1 ± 1.9	23.3 ± 0.3	21.9 ± 0.8
	A2	46.3 ± 1.2	37.0 ± 2.1	31.4 ± 2.4	33.8 ± 0.7	13.2 ± 1.2	19.6 ± 1.1
	A3	55.1 ± 2.5	39.8 ± 1.1	33.2 ± 1.4	41.2 ± 3.5	22.9 ± 2.1	21.3 ± 1.3

CD $_{(0.05)}$ E*M*C*A = NS; C*A= 1.73; E*M = 1.90. A1—Polyvinylpyrrolidone; A2—Ascorbic acid; A3—Activated charcoal; M1—Murashige and Skoog; M2—Driver and Kuniyuki Walnut medium; E1-Un-forced shoot tip; E2—Forced shoot tip; C1, 150 mg/L; C, 350 mg/L; C3, 550 mg/L. The treatments were replicated thrice containing 24 explants each. The values in the table represent mean ± SD of the treatment combinations. Data was subjected to ANOVA (see Table A2). F-Test ($p \leq 0.05$) and R Squared = 0.978 (Adjusted R Squared = 0.967).

3.3. Medium, Anti-Browning Regime and Mean Browning Score per Explant (0–4)

Table 5 shows individual effects of explant and media on mean browning score per explant (0–4) at 5% level of significance. The maximum mean browning score per explant to the tune of 1.6 was recorded in MS medium, while the minimum value (1.4) was observed in DKW medium. The maximum mean browning score per explant to the tune of 1.7 was obtained in unforced shoot tips, whereas the minimum value (1.3) was observed in forced shoot tips. The interaction effect of concentration and anti-browning agent on mean browning score per explant (0–4) was found to be significant. The highest mean browning score per explant (3.4 ± 0.2) was recorded in PVP at 150 mg/L, while the lowest value (0.6 ± 0.1) was observed in ascorbic acid at 550 mg/L.

Table 5. Influence of anti-browning regime, explant source and media on mean browning score per explant (0–4) * of walnut (*Juglans regia* L.) var. Sulaiman.

		Explant (E)					
		E1			E2		
Media (M)	**Concentration (C)**	C1	C2	C3	C1	C2	C3
M1	A1	3.4 ± 0.2	1.7 ± 0.1	1.4 ± 0.1	2.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.0
	A2	2.7 ± 0.0	1.3 ± 0.0	1.1 ± 0.0	2.1 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
	A3	2.8 ± 0.4	1.3 ± 0.1	1.2 ± 0.1	2.2 ± 0.4	0.8 ± 0.1	0.8 ± 0.2
M2	A1	3.1 ± 0.1	1.5 ± 0.1	1.2 ± 0.0	2.6 ± 0.1	0.9 ± 0.1	0.7 ± 0.0
	A2	1.9 ± 0.8	1.0 ± 0.0	1.0 ± 0.0	1.9 ± 0.0	0.7 ± 0.0	0.6 ± 0.0
	A3	2.6 ± 0.4	1.1 ± 0.0	1.1 ± 0.2	2.0 ± 0.4	0.7 ± 0.0	0.6 ± 0.1

CD $_{(0.05)}$: E*M*C*A, NS; C*A, 0.10; E, 0.20; M, 0.16. A1—Polyvinylpyrrolidone; A2—Ascorbic acid; A3- Activated charcoal; M1- Murashige and Skoog; M2—Driver and Kuniyuki Walnut medium; E1—Un-forced shoot tip; E2—Forced shoot tip; C1—(150 mg/L); C2—(350 mg/L); C3—(550 mg/L). * 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—Complete browning of the entire tissue of explant. The treatments were replicated thrice containing 24 explants each. The values in the table represent mean ± SD of the treatment combinations. Data was subjected to ANOVA (see Table A3). F-Test ($p \leq 0.05$) and R Squared = 0.955 (Adjusted R Squared = 0.933)

3.4. Medium, Anti-Browning Regime and Explant Survival Percentage

Theinfluence of four factorial interactions among anti-browning agent, concentration, source of explant and media on explant survival percentage was found to be statistically

significant. The highest explant survival percentage to the tune of 74.5 ± 2.4 per cent was recorded in forced shoot tips cultured in DKW medium supplemented with ascorbic acid @ 550 mg/L, where as the lowest explant survival percentage to the tune of 25.8 ± 1.7 per cent was observed in unforced shoot tips in MSmediumsupplemented with PVP @ 150 mg/L (Table 6, Figure 2).

Table 6. Influence of anti-browning regime, explant source and media on explant survival (%) of walnut (*Juglans regia* L.) var. Sulaiman.

		Explant (E)					
		E1			E2		
Media (M)	**Concentration (C)**	C1	C2	C3	C1	C2	C3
M1	A1	25.8 ± 1.7	42.0 ± 2.9	54.0 ± 2.8	34.0 ± 2.9	47.3 ± 2.3	59.8 ± 2.3
	A2	39.8 ±2.3	56.0 ± 2.4	65.5 ± 1.5	41.6 ± 3.1	54.9 ± 2.9	71.3 ± 1.9
	A3	31.5 ±3.1	47.7 ± 2.7	59.8 ± 4.0	39.2 ± 3.3	52.5 ± 3.8	62.8 ± 2.6
M2	A1	28.3 ±2.4	44.5 ± 3.9	57.0 ± 1.2	35.9 ± 1.0	49.2 ± 3.7	65.0 ± 3.0
	A2	37.3 ±1.3	53.5 ± 2.9	68.0 ± 0.7	44.0 ± 2.3	57.3 ± 2.2	74.5 ± 2.4
	A3	34.0 ±1.0	50.2 ± 2.0	62.2 ± 3.0	41.3 ± 1.1	54.6 ± 0.9	68.3 ± 1.6

$CD_{(0.05)}$: E*M*C*A, 2.5. A1—Polyvinylpyrrolidone; A2—Ascorbic acid; A3—Activated charcoal; M1- Murashige and Skoog; M2—Driver and Kuniyuki Walnut medium; E1-Un-forced shoot tip; E2- Forced shoot tip; C1, 150 mg/L; C2, 350 mg/L; C3, 550 mg/L. The treatments were replicated thrice containing 24 explants each. The values in the table represent mean ± SD of the treatment combinations. Data was subjected to ANOVA (see Table A4). F-Test ($p \leq 0.05$) and R Squared = 0.982 (Adjusted R Squared = 0.973).



(**a**) (**b**)

Figure 2. Survival of unforced and forced explants of walnut after anti-browning treatment: (**a**) Un-forced shoot tip in DKW medium; (**b**) Forced shoot tip in DKW medium.

4. Discussion

In plant tissue culture, browning is the process in which explants release brown exudates or phenolic compounds into the medium from its cut surfaces in due course of de-differentiation and/or re-differentiation [37,38]. Beruto et al. [39] reported the generation of phenolics by elevated activity of polyphenol oxidase (PPO). Moreover, phenylalanine ammonia lyase (PAL) enzyme converts phenylalanine to free phenolic substances for PPO [40]. Various authors have reported the involvement of PAL and PPO in the tissue browning of different plant species, such as sweet potato, cabbage, apple, pear, pineapple and herbaceous peony [41–47].

Different factors that affect browning are pretreatments, medium, subculture frequency, and temperature [48]. Charcoal adsorbs the phenols, thus causing the enzymes to be deprived of the substrates required for the generation of polymers which impart browning to the medium [49,50]. In addition, Fan et al. [51] reported the inhibition of PPO activity by NaCl, as well as by citric acid and ascorbic acid. Culture storage at low temperature was also found to enhance the activity of the PAL enzyme in walnuts [52].

A lower percentage of explant and media browning and higher survival percentage was found in the DKW medium as compared to the MS medium. The reason for this could be that the DKW medium contained a relatively lower quantity of nitrate compounds as compared to the MS medium (see Table 2), which could have induced the release of various yellowish brown phenolic exudates from the cut-off surfaces of explants [53]. These exudates, including juglone, in the medium interrupt cell growth, inhibit growth of the explant and also adversely affect their survival [26,54,55]. The superiority of the DKW medium over the MS medium could be attributed to its distinct composition of macronutrients, and also the Gelrite (gelling agent) in DKW is thought to be one of the key factors [22]. Compared to forced shoot tips, unforced shoot tips resulted in maximum percentage of explant and media browning and a lower survival percentage. This might be due to the high concentrations of phenolic compounds present in actively developing cells [56]. This variation in response may also be due to the relatively lesser inoculum load of the forced explants from dormant woods incubated in the growth chamber. However, the unforced explants from the unforced source grown in the open field conditions were exposed to the attack of different micro-organisms [57]. One more reason could be that during the process of forcing of explants in the growth chamber at a definite light and temperature regime, explants might have got acclimatized to in vitro conditions which have been otherwise found to influence polyphenol oxidase activity [58].

The higher percentage of explant survival and lower explant and media browning percentage was recorded in ascorbic acid at 550 mg/L, as compared to activated charcoal and polyvinylpyrrolidone at 50 mg/L. There could be two reasons for this. One reason is that when substrates lack PPO, ascorbic acid de-activates polyphenol oxidase (PPO) in an irreversible manner, probably through binding to its active site, preferentially in its oxy form. In the presence of PPO substrates ascorbic acid reduces PPO oxidized reaction products which results in a lag phase in PPO activity and reduces the dark product formation to a greater extent [59]. In addition, ascorbicacid at higher concentrations (350–1000 mg/L) performed as a prominent antioxidant and reduced the browning process to a greater extent [28–30]. However, the activated charcoal and PVP resulted in relatively poor results. They mainly act as adsorbents that bind phenolic compounds released from explant cultures and make them less toxic [60,61] but, at the same time, it has been observed that these compounds adsorb vitamins, growth hormones and nutrient ions, which might create nutritional and hormonal stress and result in lower percentage of survival and more browning in explants [60]. PVP has shown species specific effects in controlling the browning process [62], which may be due to the presence of a wide variety of phenolics in different plant species. The significance of anti-browning agent, concentration, explant and media on explant survival percentage might be due to the positive synergistic effects of these factors on the growth of the explants.

5. Conclusions

Based on emanated results of the present experiments it may be concluded that forced shoot tips along with DKW medium were found to be the best in terms of least browning and highest survival percentage, as compared to unforced shoot tips grown in MS medium. Anti-browning agent, ascorbic acid at 550 mg/L, showed the greatest response in terms of least browning and the highest survival percentage of explants.

Author Contributions: S.N.B., M.A.M. and A.K. has contributed to the conceptualization and experimentation of the research work; N.N., S.S.M. and M.S.D., investigated the work plan and worked on resource collection; I.K., M.A.H. and S.H.W. contributed towards computation, designing of experiments, data interpretation, discussion of results; S.N.B., M.A.M. and M.A.H. contributed to writing, editing and final preparation of manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This was purely a master's research work and has not received any external funding for this purpose.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data generated in this research is presented within this article.

Acknowledgments: S.N.B. and M.A.M. are highly thankful to Seven Star Fruits Pvt. Ltd., Jalna (MH), India, for providing the infrastructural facilities during this work.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Analysis of Variance and Tests of Between-Subject Effects for explant browning (%) of walnut (*Juglans regia* L.) var. Sulaiman.

Dependent Variable:	**Response**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	21,098.191 [b]	53	398.079	27.793	0.000
Intercept	223,149.113	1	223,149.113	15,579.546	0.000
Explant	4948.022	2	2474.011	172.727	0.000
Conc	11,944.359	2	5972.180	416.958	0.000
Media	617.566	1	617.566	43.116	0.000
AntB.A	2395.080	2	1197.540	83.608	0.000
Explant * Conc	247.855	4	61.964	4.326	0.003
Explant * Media	241.201	2	120.600	8.420	0.000
Explant * AntB.A	41.606	4	10.402	0.726	0.576
Conc * Media	5.938	2	2.969	0.207	0.813
Conc * AntB.A	247.073	4	61.768	4.312	0.003
Media * AntB.A	33.589	2	16.795	1.173	0.313
Explant * Conc * Media	33.028	4	8.257	0.576	0.680
Explant * Conc * AntB.A	256.664	8	32.083	2.240	0.030
Explant * Media * AntB.A	42.545	4	10.636	0.743	0.565
Conc * Media * AntB.A	23.081	4	5.770	0.403	0.806
Explant * Conc * Media * AntB.A	20.584	8	2.573	0.180	0.993
Error	1546.907	108	14.323		
Total	245,794.210	162			
Corrected Total	22,645.097	161			

Table A2. Analysis of Variance and Tests of Between-Subject Effects for media browning (%) of walnut (*Juglans regia* L.) var. Sulaiman.

Dependent Variable:	**Response**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	16,551.497 [b]	53	312.292	90.727	0.000
Intercept	162,114.507	1	162,114.507	47097.576	0.000
Explant	3277.921	2	1638.960	476.151	0.000
Conc	10,735.006	2	5367.503	1559.369	0.000
Media	73.744	1	73.744	21.424	0.000
AntB.A	1282.382	2	641.191	186.279	0.000
Explant * Conc	304.279	4	76.070	22.100	0.000
Explant * Media	2.943	2	1.472	.428	0.653
Explant * AntB.A	80.665	4	20.166	5.859	0.000
Conc * Media	0.003	2	0.002	.001	0.999
Conc * AntB.A	561.103	4	140.276	40.753	0.000
Media * AntB.A	0.005	2	0.002	.001	0.999
Explant * Conc * Media	0.005	4	0.001	0.000	1.000
Explant * Conc * AntB.A	233.412	8	29.176	8.476	0.000
Explant * Media * AntB.A	0.006	4	0.001	0.000	1.000
Conc * Media * AntB.A	0.007	4	0.002	0.000	1.000
Explant * Conc * Media * AntB.A	0.016	8	0.002	0.001	1.000
Error	371.747	108	3.442		
Total	179,037.750	162			
Corrected Total	16,923.243	161			

Table A3. Analysis of Variance and Tests of Between-Subject Effects for mean browning score per explant (0–4) of walnut (*Juglans regia* L.) var. Sulaiman.

Dependent Variable:	**Response**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	102.860 [b]	53	1.941	43.261	0.000
Intercept	182.384	1	182.384	4065.521	0.000
Explant	6.490	2	3.245	72.333	0.000
Conc	86.070	2	43.035	959.293	0.000
Media	1.566	1	1.566	34.918	0.000
AntB.A	6.491	2	3.246	72.348	0.000
Explant * Conc	0.040	4	0.010	0.220	0.927
Explant * Media	0.088	2	0.044	0.984	0.377
Explant * AntB.A	0.120	4	0.030	0.669	0.615
Conc * Media	0.138	2	0.069	1.534	0.220
Conc * AntB.A	1.363	4	0.341	7.597	0.000
Media * AntB.A	0.015	2	0.008	0.173	0.842
Explant * Conc * Media	0.066	4	0.017	0.369	0.831
Explant * Conc * AntB.A	0.124	8	0.016	0.346	0.946
Explant * Media * AntB.A	0.081	4	0.020	0.453	0.770
Conc * Media * AntB.A	0.104	4	0.026	0.579	0.679
Explant * Conc * Media * AntB.A	0.103	8	0.013	0.287	0.969
Error	4.845	108	0.045		
Total	290.089	162			
Corrected Total	107.705	161			

Table A4. Analysis of Variance and Tests of Between-Subject Effects for explant survival (%) of walnut (*Juglans regia* L.) var. Sulaiman.

Dependent Variable:	**Response**				
Source	**Type III Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Corrected Model	45,953.049 [b]	53	867.039	108.541	0.000
Intercept	410,881.197	1	410,881.197	51,436.749	0.000
Explant	11,824.454	2	5912.227	740.131	0.000
Conc	30,550.312	2	15,275.156	1912.242	0.000
Media	88.002	1	88.002	11.017	0.001
AntB.A	466.952	2	233.476	29.228	0.000
Explant * Conc	1710.347	4	427.587	53.528	0.000
Explant * Media	104.749	2	52.375	6.557	0.002
Explant * AntB.A	77.474	4	19.369	2.425	0.052
Conc * Media	225.480	2	112.740	14.114	0.000
Conc * AntB.A	60.325	4	15.081	1.888	0.118
Media * AntB.A	142.223	2	71.112	8.902	0.000
Explant * Conc * Media	102.654	4	25.664	3.213	0.016
Explant * Conc * AntB.A	135.182	8	16.898	2.115	0.040
Explant * Media * AntB.A	216.921	4	54.230	6.789	0.000
Conc * Media * AntB.A	9.204	4	2.301	.288	0.885
Explant * Conc * Media *AntB.A	238.769	8	29.846	3.736	0.001
Error	862.713	108	7.988		
Total	457,696.960	162			
Corrected Total	46,815.763	161			

References

1. Dandekar, A.; Leslie, C.; McGranahan, G. *Juglans regia* Walnut. In *Biotechnology of Fruit and Nut Crops*; Biotechnology in Agriculture 29; Litz, R.E., Ed.; The Doyle Foundation: Glasgow, UK, 2005.
2. Bernard, A.; Lheureux, F.; Dirlewanger, E. Walnut: Past and future of genetic improvement. *Tree Genet. Genomes* **2017**, *14*, 1–28. [CrossRef]
3. Pollegioni, P.; Woeste, K.; Chiocchini, F.; Del Lungo, S.; Ciolfi, M.; Olimpieri, I.; Clark, J.; Hemery, G.E.; Mapelli, S.; Malvolti, M.E.; et al. Rethinking the history of common walnut (*Juglans regia* L.) in Europe: Its origins and human interactions. *PLoS ONE* **2017**, *12*, e0172541. [CrossRef] [PubMed]
4. Aradhya, M.K.; Potter, D.; Gao, F.; Simon, C.J. Molecular phylogeny of *Juglans*(Juglandaceae): A biogeographic perspective. *Tree Genet. Genomes* **2007**, *3*, 363–378. [CrossRef]
5. McGranahan, G.; Leslie, C. Breeding walnuts (*Juglans regia*). In *Breeding Plantation Tree Crops: Temperate Species*; Mohan Jain, S., Priyadarshan, P.M., Eds.; Springer: New York, NY, USA, 2009.
6. Vahdati, K.; Ashrafi, E.N.; Ebrahimzadeh, H.; Mirmasoumi, M. Improved Micropropagation of Walnut (*Juglans regia* L.) on Media Optimized for Growth Based upon Mineral Content of Walnut Seed. 1st International Symposium on Biotechnology of Fruit Species. *Acta Hortic.* **2009**, *839*, 117–124.
7. Kaur, R.; Sharma, N.; Kumar, K.; Sharma, D.R.; Sharma, S.D. In vitro germination of walnut (*Juglans regia* L.) embryos. *Sci. Hortic.* **2006**, *109*, 385–388. [CrossRef]
8. FAO. 2020. Available online: http://www.fao.org/faostat (accessed on 10 August 2022).
9. Rezaee, R.; Vahdati, K.; Grigoorian, W.; Valizadeh, M. Walnut grafting success and bleeding rate as affected by different grafting methods and seedling vigor. *J. Hortic. Sci. Biotechnol.* **2008**, *83*, 94–99. [CrossRef]
10. Hassan, G.I.; Bhat, Z.A.; Sofi, J.A.; Wani, A.A.; Sofi, K.A.; Wani, A.W.; Khan, O.A.; Dar, A.A.; John, I.; Hajam, M.A. Propagation of Persian walnut (*Juglans regia* L.) under controlled climatic conditions. *J. Pharmacogn. Phytochem.* **2019**, *8*, 1675–1677.
11. McGranahan, G.; Leslie, C. Walnuts (*Juglans*). *Acta Hortic.* **1991**, *290*, 907–974. [CrossRef]
12. Sanchez-Zamora, M.A.; Cos-Terrer, J.; Frutos-Tomas, D. Embryo germination and proliferation in vitro of *Juglans regia* L. *Sci. Hortic.* **2006**, *108*, 317–321. [CrossRef]
13. Cummins, J.N.; Ashby, W.C. Aseptic culture of *Juglans nigra* stem tissues. *For. Sci.* **1969**, *15*, 102–103.
14. McGranahan, G.H.; Driver, J.A.; Tulecke, W. Tissue culture of *Juglans*. In *Cell and Tissue Culture in Forestry*; Bonga, J.M., Durzan, D.J., Eds.; Martinus Nijhoff: Boston, MA, USA, 1987; Volume 3, pp. 261–271.

15. Cornu, D.; Jay-Allemand, C. Micropropagation of hybrid walnut trees (*Juglans nigra* × *Juglans regia*) through culture and multiplication of embryos. In *Annales des Sciences Forestières*; Dreyer, E., Ed.; Elsevier: Amsterdam, The Netherlands, 1989; Volume 46.
16. Pijut, P.M. Micropropagation of *Juglans cinerea* L. (butternut). In *High-Tech and Micropropagation V. Biotechnology in Agriculture and Forestry*; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany, 1997; Volume 39, pp. 345–357.
17. Navatel, J.C.; Bourrain, L. Plant production of walnut *Juglans regia* L. by in vitro multiplication. *Acta Hortic.* **2001**, *544*, 465–471. [CrossRef]
18. Gotea, R.; Gotea, I.; Sestras, R.E.; Vahdati, K. In vitro propagation of several walnut cultivars. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. *Horticulture* **2012**, *69*, 167–171.
19. Licea-Moreno, R.J.; Contreras, A.; Morales, A.V.; Urban, I.; Daquinta, M.; Gomez, L. Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA. *Plant Cell Tissue Organ Cult.* **2015**, *123*, 143–154. [CrossRef]
20. Payghamzadeh, K.; Kazemitabar, S.K. In vitro propagation of walnut—A review. *Afr. J. Biotechnol.* **2011**, *10*, 290–311.
21. Toosi, S.; Dilmagani, K. Proliferation of *Juglans regia* L. by in vitro embryo culture. *J. Cell Biol. Genet.* **2010**, *1*, 12–19.
22. Rahman, S.S. DKW emerges as a superior media factor in in vitro plant regeneration. *J. Agrisearch* **2018**, *1*, 3–4.
23. Yegizbayeva, T.K.; García-García, S.; Yausheva, T.V.; Kairova, M.; Apushev, A.K.; Oleichenko, S.N.; Licea-Moreno, R.J. Unraveling Factors Affecting Micropropagation of Four Persian Walnut Varieties. *Agronomy* **2021**, *11*, 1417. [CrossRef]
24. Ehteshamnia, A.; Gholami, M. Inhibition of Persian walnut (*Juglans regia* L.) microcuttings browning by utilizing different methods. *J. Biodivers. Environ. Sci.* **2014**, *5*, 562–571.
25. Xiao-Dong, C.A.I.; Wang, G.Y.; Wen-Juan, C.A.O. In vitro induction and proliferation of callus from immature cotyledons and embryos of *Juglans regia* cv. 'Xiangling'. *NotulaeBotanicae Horti Agrobotanici Cluj-Napoca* **2013**, *41*, 378–384.
26. Rout, G.R.; Samantaray, S.; Mottley, J.; Das, P. Biotechnology of the rose: A review of recent progress. *Sci. Hortic.* **1999**, *81*, 201–228. [CrossRef]
27. Christiansen, J.; Fonnesbech, M. Prevention by polyvinylpyrrolidone of growth inhabitation of *Hamamelis* shoot tips grown in vitro and browning of the agar medium. *Acta Hortic.* **1975**, *54*, 101–104. [CrossRef]
28. Babaei, N.; Abdullah, N.A.P.; Saleh, G.; Abdullah, T.L. Control of contamination and explant browning in *Curculigo latifolia* in vitrocultures. *J. Med. Plants Res.* **2013**, *7*, 448–454.
29. Gioushy, S.F.E.; Liu, R.; Fan, H.K. A complete protocol to reduce browning during coconut (*cocos nucifera* L.) tissue culture through shoot tips and inflorescence explants. *Plant Arch.* **2020**, *20*, 2196–2204.
30. Mir, J.I.; Ahmed, N.; Itoo, H.; Sheikh, M.A.; Wani, S.H.; Rashid, R.; Mir, H. Technique to minimize phenolics in walnut in in vitro culture initiation. *Indian J. Hortic.* **2017**, *74*, 285–287. [CrossRef]
31. Poudyal, B.K.; Du, G.; Zhang, Y.; Liu, J.; Shi, Q. Studies on browning problem and phenols content on shoots of Yali, Aikansui and Abbe Fetel pears for in vitro culture. *Front. Agric. China* **2008**, *2*, 321–330. [CrossRef]
32. Dalal, M.A.; Sharma, B.B.; Rao, M.S. Studies on stock plant treatment and initiation culture mode in control of oxidative browning in in vitroculture of grape vine. *Sci. Hortic.* **1992**, *51*, 35–41. [CrossRef]
33. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiol.* **1962**, *15*, 473–497. [CrossRef]
34. Driver, J.A.; Kuniyuki, A.H. In vitropropagation of paradox walnut rootstock. *J. Hortic. Sci.* **1984**, *19*, 507–509.
35. Dodds, J.H.; Roberts, L.W. *Experiments in Plant Tissue Culture*; Cambridge University Press: London, UK, 1982; p. 178.
36. Anderson, W.C. Propagation of *Rhododendrons* by tissue culture. Part I. Development of a culture medium for multiplication of shoots. *Proc. Int. Plant Propag. Soc.* **1975**, *25*, 129–135.
37. George, E.F.; Hall, M.A.; Klerk, G.J.D. *Effects of the Physical Environment: Plant Propagation by Tissue Culture*; Wiley-Blackwell: Dordrecht, The Netherlands, 2008; Volume 1, pp. 423–464.
38. Shen, H.L. *Plant Tissue Culture*; China's Forestry Press: Beijing, China, 2005; Volume 3, pp. 256–261. (In Chinese)
39. Beruto, M.; Curir, P.; Debergh, P. Callus growth and somatic embryogenesis in thalamus tissue of *Ranunculus asiaticus* L. cultivated in vitro: Cytokinin effect and phenol metabolism. *In Vitro Cell. Dev. Biol. Anim.* **1996**, *32*, 154–160.
40. Camm, E.L.; Towers, G.H.N. Phenylalanine ammonia lyase. *Phytochemistry* **1977**, *12*, 961–973. [CrossRef]
41. Murata, M.; Nishimura, M.; Murai, N.; Haruta, M.; Homma, S.; Itoh, Y.J. A transgenic apple callus showing reduced polyphenol oxidase activity and lower browning potential bioscience. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 383–388. [CrossRef] [PubMed]
42. Zhou, Y.C.; Dahler Janelle, M.; Underhill Steven, J.R.; Wills Ron, B.H. Enzymes associated with blackheart development in pineapple fruit. *Food Chem.* **2003**, *80*, 565–572. [CrossRef]
43. Liao, Z.; Chen, R.; Chen, M.; Yang, Y.; Fu, Y.; Zhang, Q.; Lan, X. Molecular cloning and characterization polyphenol oxidase gene from sweetpotato. *Mol. Biol.* **2006**, *40*, 907–913. [CrossRef]
44. Yan, S.J.; Li, L.; He, L.H.; Liang, L.Y.; Li, X.D. Maturity and cooling rate affects browning: Polyphenol oxidase activity and gene expression of 'Yali' pears during storage. *Postharvest Biol. Technol.* **2013**, *85*, 39–44. [CrossRef]
45. Cheng, Y.D.; Liu, L.Q.; Zhao, G.Q.; Shen, C.G.; Yan, H.B.; Guan, J.F.; Yang, K. The effects of modified atmosphere packaging on core browning and the expression patterns of PPO and PAL genes in 'Yali' pears duriing cold storage. *LWT-Food Sci. Technol.* **2015**, *60*, 11243–11248. [CrossRef]
46. Banerjee, A.; Penna, S.; Variyar, P.S.; Sharma, A. Gamma irradiation inhibits wound induced browning in shredded cabbage. *Food Chem.* **2015**, *173*, 38–41. [CrossRef]

47. Cai, X.; Wei, H.; Liu, C.; Ren, X.; Thi, L.T.; Jeong, B.R. Synergistic Effect of NaCl Pretreatment and PVP on Browning Suppression and Callus Induction from Petal Explants of *Paeonia Lactiflora* Pall. 'Festival Maxima'. *Plants* **2020**, *9*, 346. [CrossRef]
48. Tian, D. Container Production and Post-Harvest Handling of Lotus (Nelubo) and Micropropagation of Herbaceous Peony (Paeonia). Ph.D. Thesis, Auburn University, Auburn, AL, USA, 2008; pp. 56–58.
49. Johansson, L. Effects of activated charcoal in anther cultures. *Physiol. Plant.* **1983**, *59*, 397–403. [CrossRef]
50. Fridborg, G.; Pedersen, M.; Landstrom, L.E.; Eriksson, T. The effect of activated charcoal on tissue cultures: Adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant.* **1978**, *43*, 104–106. [CrossRef]
51. Fan, M.H.; Wang, M.; Zou, P.B. Effect of sodium chloride on the activity and stability of polyphenol oxidase from fuji apple. *J. Food Biochem.* **2005**, *29*, 221–230. [CrossRef]
52. Christopoulos, M.V.; Tsantili, E. Participation of phenylalanine ammonia-lyase (PAL) in increased phenolic compounds in fresh cold stressed walnut (*Juglans regia* L.) kernels. *Postharvest Biol. Technol.* **2015**, *104*, 17–25. [CrossRef]
53. Cheema, G.S.; Sharma, D.P. In vitro propagation of apple root-stock-EMLA 25. *Acta Hortic.* **1983**, *131*, 75–88. [CrossRef]
54. Yu, D.H.; Meredith, C.P. The influence of explant origin on tissue browning and shoot production in shoot tip culture of grapevine. *J. Am. Soc. Hortic. Sci.* **1986**, *111*, 972–975. [CrossRef]
55. Esau, K. *Anatomy of Seed Plants*, 2nd ed.; John Wiley and Sons, Inc.: New York, USA, 1977; pp. 111–119.
56. Dalal, M.A.; Das, B.; Sharma, A.K.; Mir, M.A.; Sounduri, A.S. In vitro cloning of apple (*Malus domestica* Borkh) employing forced shoot tip cultures of M9 rootstock. *Indian J. Biotechnol.* **2006**, *5*, 543–550.
57. Torres, K.; Carlisi, J.A. Shoot and root organogenesis of *Camellia sasanqua*. *Plant Cell Rep.* **1986**, *5*, 381–384. [CrossRef]
58. Pittet, H.; Moncousin, C. Multiplication novelle du rosier. *Rev. Hortic. Sci.* **1981**, *54*, 169–173.
59. Arias, E.; Gonzalez, J.; Peiro, J.M.; Oria, R.; Buesa, P.L. Browning prevention by ascorbic acid and 4-hexylresorcinol: Different machanisms of action on polyphenols oxidase in the presence and in the absence of substrates. *J. Food Sci.* **2007**, *72*, 464–470. [CrossRef]
60. Thomas, T.D. Role of activated charcoal in plant tissue culture. *Biotechnol. Adv.* **2008**, *26*, 618–631. [CrossRef]
61. Uchendu, E.E.; Paliyath, G.; Brown, D.C.W.; Saxena, P.K. In vitro propagation of North American ginseng (*Panax quinquefolius* L.). *In Vitro Cell. Dev. Biol. Plant* **2011**, *47*, 710–718. [CrossRef]
62. Chevere, A.M.; Gill, S.S.; Mouras, A.; Salesses, G. In vitro vegetative multiplication of chestnut. *J. Hortic. Sci.* **1983**, *58*, 23–29. [CrossRef]