Optimization of conditions for efficient *in vitro* culture establishment of *Terminalia arjuna*, an important medicinal plant of the Indian sub-continent

Rani Anita[1], Chahal Shiwani[1], Singh Inderjeet[1], Gulia Vibhuti[2] and Siwach Priyanka[1*]
1. Department of Biotechnology, Chaudhary Devi Lal University, Sirsa-125055, INDIA
2. Department of Botany, Chaudhary Devi Lal University, Sirsa-125055, INDIA
*priyankasiwach@cdlu.ac.in; psiwach29@gmail.com

Abstract
The in vitro propagation of large medicinal tree species like Terminalia arjuna is very difficult because of many problems from the collection of explants to the culture establishment. The dominant challenges are microbial contamination and phenolic browning. These are resolved by implementing specific treatments before and during explant collection and inoculation. Various surface sterilizing agents were utilized to remove microbial contamination. Accurate knowledge about the concentration and treatment time of the antimicrobial agents is important as their higher dosage can lead to unfavorable consequences on the in vitro cultured plantlets. Different efforts have been made to control phenolic browning, such as presoaking the explants in chilled antioxidant solution, adding antioxidants into the culture medium and other conventional practices like frequent subculturing the explants and keeping the cultured vessels in dark conditions. The dosage of the antimicrobials and antioxidants depended on the season in which the explant was collected.

During the growing season of the selected mother tree, the best antimicrobial treatment was found to be mild whereas the best antioxidant treatment was strong because in this season, endophytic microbes are less prominent and there was heavy leaching of phenolic substances from the cut ends of the explants. Further, the mother tree was lopped during October to eliminate contamination due to recalcitrant microbes in aged tissues.

Keywords: *In vitro* propagation, culture establishment, contamination, phenolic browning, endophytic microbes.

Introduction

Terminalia arjuna (Roxb.) Wight and Arn., popularly known as Arjuna, a member of the Combretaceae family, is a large evergreen tree (having 20-26 m height and 3 m girth), native to the Indian sub-continent[6]. Almost all parts of the tree (like flowers, fruits, roots, wood, bark and leaves) are useful for medicinal, household and industrial purposes[2]. It has antimutagenic, antibacterial, antioxidant, hypolipidemic, anti-inflammatory and hypocholesterolemic effects[25]. *T. arjuna*'s main active constituents are cardenolide, gallic acid, oligomeric proanthocyanidines (e.g., OPCs), triterpenoid saponins (e.g. arjunolic acid, arjunic acid, arjungenin, arjunglycosides), ellagic acid, flavonoids (e.g., arjunolone, arjunone, luteolin), phytosterols, tannins, magnesium, calcium, copper and zinc[1,16,18].

The bark of arjuna is widely used in the treatment of heart diseases like coronary artery disease, mitral regurgitation, congestive heart failure, cardiomyopathy, ventricular failure and heart attack[13]. Regular use of *T. arjuna* bark improves cardiac muscle strength and pumping activity of the heart, so the bark is used as an ayurvedic remedy for cardiovascular disorders[8].

Though *T. arjuna* can easily be found across the Indian subcontinent but due to its overexploitation by the herbal medicinal industry and timber industry, its natural population is decreasing at an alarming rate. So, to replenish its speedy loss and to meet the medicinal requirements, its large-scale propagation is the need of the hour. However, the conventional propagation methods in arjuna have certain drawbacks such as low seed germination due to the hard seed coat and difficulty in rooting by air layering and cuttings.

Therefore, there is a crucial urgency for applying non-conventional propagation techniques for mass production. Micropropagation is an important method for such medicinal and economically useful trees. Propagation of large trees through micropropagation has many advantages over conventional propagation techniques: the production of true-to-type plants, rapid multiplication of selected genotypes, season-independent propagation, production of disease-free plants, requiring less space when compared to the seed-grown saplings[7].

Plant tissue culture methods in perennial trees are severely hampered due to high microbial contamination and excessive phenolic browning and both these factors are also influenced by seasonal changes[6]. Successful culture establishment is essential because it reduces time, labour and materials, preventing significant economic losses. The *in vitro* growing plants are considered to be under stress and they are very susceptible to direct infection, even by non-pathogenic microbes[10].

The possibility of contamination occurs mainly due to two factors: first, it can be from the selected explants where the microbes tend to remain inactive for a longer period of time and second, due to improper handling of the operator[15].

During contamination, competition occurs between plants and microbes for the nutrients present in the culture media, leading to the death of the plantlets which are very fragile when grown in controlled conditions. Microbial contamination can be controlled by using various methods such as cryotherapy, thermotherapy, chemotherapy and by utilizing various surface sterilizing agents such as sodium hypochlorite, mercuric chloride etc.[17]

Another major problem i.e. phenolic browning of plant tissues causes unwanted modifications in the explants and can cause the death of the explants, thus impeding the successful *in vitro* propagation[3]. Phenolic substances are released from the wounded parts of the explants as a defence mechanism in response to stress. Generally, the browning can be overcome by removing phenolic compounds by adding the anti-browning compounds into the medium, immersing the explants in anti-browning solutions etc.[19]

The remarkable impact of seasonal variations on microbial contamination and phenolic browning was also studied. When the explants were collected during the growing season of the mother tree, the frequency of microbial contamination was less than in other seasons when the tissues were in dormant stages. Meanwhile, the phenolic compounds were released more vigorously during the growing season than in other periods of the year.

The cultural establishment of *Terminalia arjuna* has been found to be a key challenge in establishing an efficient micropropagation protocol for this important medicinal tree[6]. The present research work was targeted to ample experimentation towards optimizing conditions for enhanced culture establishment of selected explants of *Terminalia arjuna.*

Material and Methods

Plant Materials: Two explants (shoot tips and nodal segments) were collected from *T. arjuna* (Roxb.) Wight and Arn trees are located at Chaudhary Devi Lal University, Sirsa, Haryana, India. To authenticate the *T. arjuna* trees, voucher specimens were deposited (Acc. No. 1215-1219) at the Northern Regional Centre of Botanical Survey of India, Dehra Dun, India, which were confirmed via a certificate sent by email with identity no.: BSI/NRC Tech./ Herb(Ident.)/2022-23/608 dated 18 Nov., 2022.

Culture media conditions: Collected explants were cultured in 300 ml jam bottles containing 50 ml modified Murashige and Skoog (MS) medium. The pH of the culture media was calibrated between 5.7 and 5.8 before autoclaving at 121°C temperature and 15 psi pressure for 15 min. Then, the cultured vessels were kept at 25±5°C temperature and installed on culture racks in a designated culture room with 16 hours of light and 8 hours of dark conditions.

Optimizing conditions for Culture establishment: Culture establishment here refers to the situation where the green, healthy state of explants is observed after about 4-5 weeks of culture. The 'Culture Establishment' experiments were targeted towards two things: first, to control the microbial contamination of the cultures and second, to minimize the damage caused by the release of phenolic substances from the cut end of explants. The details are given as follows:

(i) Effect of various surface sterilizing agents: To control microbial contamination, 15 surface sterilizing treatments (SST) were designed having different concentrations and combinations of various sterilizing agents such as $HgCl_2$, NaOCl, bavistin, alcohol etc. (Table 1).

(ii) Effect of various anti-oxidant (AO) solutions: For minimizing the effects of phenolic browning, different absorbents and antioxidants were used. The collected explants were treated with chilled solution (4°C) having different concentrations of citric acid, ascorbic acid and polyvinylpolypyrrolidone (PVPP) for different periods. Henceforth, 8 anti-oxidant (AO) treatments were designed for treating the explants, as shown in table 2.

Table 1
Different surface sterilizing treatments given to explants to control microbial contamination.

Treatment Number	Tween-20	Bavistin	NaOCl	HgCl2	Alcohol
SST-1	2% (10min)	0.1%(10min)	4% (6min)	-	70%(30s)
SST-2	2% (10min)	0.1%(12min)	4% (8min)	-	70%(30s)
SST-3	2% (10min)	0.1%(12min)	5% (4min)	-	70%(30s)
SST-4	2% (10min)	0.1%(10min)	5% (6min)	-	70%(30s)
SST-5	2% (10min)	0.1%(8min)	-	0.1%(7min)	70%(30s)
SST-6	2% (10min)	0.1%(10min)	-	0.1%(8min)	70%(30s)
SST-7	1% (15min)	0.1%(12min)	-	0.1%(8min)	70%(30s)
SST-8	2% (10min)	0.1%(10min)	-	0.1%(8min)	70%(50s)
SST-9	2% (10min)	0.1%(8min)	-	0.1%(8min)	50%(60s)
SST-10	1% (10min)	0.1%(10min)		0.1%(8min)	70%(60s)

Abbreviations: NaOCl-Sodium Hypochlorite, $HgCl_2$-Mercuric Chloride

Table 2
Various anti-oxidant solutions used for controlling phenolic exudation from cut portions of explant.

Treatment Code	Antioxidant treatment to explants			
	Composition (mg/L)			Time Duration
	Ascorbic acid	Citric acid	PVPP	
AO-1	100	50	25	20min
AO-2	100	50	25	30min
AO-3	100	50	25	40min
AO-4	75	35	25	30min
AO-5	75	35	25	40min

Table 3
Various pre-treatments given to shoot tip and nodal explant for controlling microbial contamination and phenolic browning with their respective CEF.

Treatment Code	Combinations of anti-oxidants and surface sterilising treatments	Culture Establishment Frequency (%) (Mean±S.E.)		
		Seasonal Division I	Seasonal Division II	
			Unlopped Mother Tree	Lopped Mother Tree
PTP-1	AO-1 + SST-1	18.53^{n} ±0.47	14.43^{s}±0.45	23.22^{o}±0.76*
PTP-2	AO-2 + SST-1	20.29^{n} ± 0.69	20.85^{q}±0.70	25.34^{o}±0.85
PTP-3	AO-3 + SST-1	19.64^{n} ±0.74	18.19^{r}±0.62	25.11^{o}±0.81
PTP-4	AO-4 + SST-1	24.70^{m} ±0.57	22.59^{q}±0.56	25.85^{o}±0.94
PTP-5	AO-5 + SST-1	23.62^{m} ±0.72	17.12^{r}±0.48	25.90^{o}±0.67
PTP-6	AO-1 + SST-2	10.85^{q} ±0.43	07.85^{u}±0.41	15.47^{q}±0.65
PTP-7	AO-2 + SST-2	16.09^{o} ±0.96	13.51^{st}±0.55	18.69^{p}±0.60
PTP-8	AO-3 + SST-2	09.00^{r} ±0.43	07.44^{uv}±0.33	11.55^{r}±0.76
PTP-9	AO-4 + SST-2	16.28^{o} ±0.30	12.01^{t}±0.79	20.40^{p}±1.00
PTP-10	AO-5 + SST-2	13.97^{p}±0.43	11.40^{t}±0.62	15.55^{q}±0.62
PTP-11	AO-1 + SST-3	04.87^{v}±0.74	03.53^{x}±0.26	6.20^{t}±0.30
PTP-12	AO-2 + SST-3	05.25^{uv}±0.74	4.66^{wx}±0.29	8.35^{st}±0.27
PTP-13	AO-3 + SST-3	6.05^{uv}±0.69	$0 3.29^{x}$±0.29	8.10^{st}±0.38
PTP-14	AO-4 + SST-3	5.92^{uv}±0.59	07.55^{uv}±3.48	11.23^{r}±0.46
PTP-15	AO-5 + SST-3	5.58^{uv}±0.50	3.57^{x}±0.26	9.39^{rs}±0.49
PTP-16	AO-1 + SST-4	6.50^{tuv}±0.46	6.33^{uvw}±0.36	10.77^{rs}±0.62
PTP-17	AO-2 + SST-4	7.00^{stu}±0.49	6.62^{uvw}±0.28	9.45^{rs}±0.50
PTP-18	AO-3 + SST-4	6.50^{tuv}±0.47	5.35^{uvwx}±0.24	8.40^{st}±0.63
PTP-19	AO-4 + SST-4	8.40^{rs}±0.42	6.44^{uvw}±0.40	10.90^{rs}±0.44
PTP-20	AO-5 + SST-4	8.13^{rst}±0.49	5.30^{vwx}±0.35	10.63^{rs}±0.67
PTP-21	AO-1 + SST-5	57.39^{h}±0.31	52.73^{jk}±0.36	74.06^{fg}±1.05
PTP-22	**AO-2 + SST-5**	77.28^{b}±0.36	61.04^{fgh}±0.51	**94.68^{a}±0.90**
PTP-23	AO-3 + SST-5	78.30^{b}±0.41	60.49^{gh}±0.70	91.50^{bc}±1.22
PTP-24	AO-4 + SST-5	65.59^{de}±0.64	64.82^{de}±0.42	71.05^{hi}±0.88
PTP-25	**AO-5 + SST-5**	**83.31^{a}±0.91**	60.80^{fgh}±0.49	93.09^{ab}±0.96
PTP-26	AO-1+ SST-6	65.44^{e}±0.81	51.00^{k}±0.83	71.22^{ghi}±0.72
PTP-27	AO-2+ SST-6	67.83^{d}±0.44	62.59^{efg}±0.50	91.61^{bc}±1.05
PTP-28	AO-3+ SST-6	72.30^{c}±0.58	60.20^{ghi}±0.44	88.44^{de}±1.37
PTP-29	AO-4+ SST-6	60.17^{g}±0.75	63.06^{ef}±0.67	86.98^{e}±1.34
PTP-30	AO-5+ SST-6	67.17^{de}±0.53	57.98^{i}±0.66	88.90^{cde}±1.21
PTP-31	AO-1 + SST-7	61.82^{fg}±0.63	66.77^{cd}±0.68	73.35^{fgh}±0.36
PTP-32	AO-2+ SST-7	65.69^{de}±0.55	67.15^{c}±0.47	90.25^{cd}±1.14
PTP-33	AO-3+ SST-7	70.05^{d}±0.87	63.95^{e}±0.65	62.62^{kl}±1.14
PTP-34	**AO-4+ SST-7**	63.32^{f}±0.49	**74.04^{a}±0.57**	87.90^{de}±0.87
PTP-35	AO-5+ SST-7	73.03^{c}±0.50	69.72^{b}±0.67	88.96^{cde}±0.93
PTP-36	AO-1+ SST-8	55.48^{i}±0.65	59.85^{hi}±0.60	64.86^{jk}±0.73
PTP-37	AO-2+ SST-8	65.60^{de}±0.47	60.80^{fgh}±0.62	70.84^{i}±0.87

PTP-38	AO-3+ SST-8	66.33^{de}±0.45	53.31^{j}±0.55	71.31^{ghi}±1.06
PTP-39	AO-4+ SST-8	61.89^{fg}±0.68	61.03^{fgh}±0.86	71.45^{ghi}±0.98
PTP-40	AO-5+ SST-8	40.42^{l}±0.58	34.59^{p}±0.47	50.07^{n}±0.79
PTP-41	AO-1+ SST-9	51.76^{j}±0.82	42.85^{m}±0.79	60.65^{l}±0.76
PTP-42	AO-2+ SST-9	51.09^{j}±0.76	46.58^{l}±0.99	60.60^{l}±1.02
PTP-43	AO-3+ SST-9	45.00^{k}±0.54	36.60^{op}±1.36	55.12^{m}±0.84
PTP-44	AO-4+ SST-9	46.60^{k}±0.43	39.79^{n}±0.91	57.27^{m}±1.01
PTP-45	AO-5+ SST-9	41.08^{l}±0.50	37.72^{no}±1.01	50.46^{n}±0.93
PTP-46	AO-1+ SST-10	56.60^{hi}±0.49	34.84^{p}±0.58	66.45^{j}±1.00
PTP-47	AO-2+ SST-10	66.20^{de}±0.47	42.20^{m}±0.74	73.66^{fghi}±0.72
PTP-48	AO-3+ SST-10	67.39^{d}±0.41	44.32^{m}±0.88	72.50^{fghi}±0.93
PTP-49	AO-4+ SST-10	62.44^{f}±0.66	36.00^{op}±0.42	73.04^{fghi}±0.80
PTP-50	AO-5+ SST-10	69.90^{d}±0.81	36.32^{op}±0.37	75.10^{f}±1.30

*Mean separation by Duncan Multiple Range Test with their respective standard error. The mean value associated with the same superscript letter in a column are not significantly different

Influence of season on culture establishment: For this study, one full year was divided into two seasonal divisions (based on the variations in local weather conditions): seasonal division-I was from April to September and seasonal division II was from October to March, to find out the best conditions supporting maximum CEF in each seasonal division. Seasonal division- I corresponds to the active period of growth for the selected plant in North India while during seasonal division- II, the plant has aged tissue that contains endophytic microorganisms which cause heavy contamination of nutrient media. During this season, explants are dormant; hence, they exhibit poor bud break response and are very difficult to sterilize due to endophytic microbes in aged tissues.

Therefore, to overcome the problem of poor bud break and heavy contamination in the seasonal division- II, the mother tree was lopped and its effect on culture establishment was also observed. The lopping of the mother tree affects the bud breaking and positively shoots induction as the unlopped tree has aged tissues that block the activation of meristem and has recalcitrant microbes. The mother tree was lopped during the month of October and produced fresh sprouts from November month.

Calculation of Culture establishment frequency (CEF): For each explant. CEF was calculated after five weeks of culture as per the following formula:

$$\text{Culture establishment frequency (CEF)} = \frac{\text{No. of healthy explants} \times 100}{\text{Total no. of cultured explants}}$$

Results and Discussion

During the culture establishment, the main hindrance was phenolic exudation as the cut ends of the explants exhibited browning in the nutrient media. Browning of the culture media occurs when the enzyme polyphenol oxidase is responsible for the oxidation of phenolic substances released by cut ends of the explants. Due to this, the nutrients were unavailable to the cultured explants; hence, the explants did not survive and eventually, the entire explant died. Eight anti-browning treatments with different concentrations of ascorbic acid, citric acid and PVPP in solutions, in which explants were incubated (in chilled antioxidant solution) for different time durations, were designed to combat this problem.

Another major challenge to the culture establishment of explants was high prevalence of microbial contamination. Various chemical and physical methods have been reported to control different kinds of contamination viz. caused by bacteria, fungi, viruses, yeasts, mites etc.[6] The chemicals include using disinfectants like mercuric chloride, sodium hypochlorite, alcohol, hydrogen peroxide and commercial bleach. Many anti-fungal agents like bavistin, captan and chlorothalonil are also used routinely during surface sterilization of various explants. For some specific contaminants, certain specific antibiotics like streptomycin, fluconazole, tetracycline etc. were also added in culture medium[14].

For each of the seasonal divisions, the 50 pre-treatment protocols were designed to control browning as well as microbial contamination and their effect on the culture establishment frequency was noted.

Seasonal division I (April to September): This period is the growing period of woody perennials in Northern India, so there are fresh sprouts on the trees. Hence, when the selected explants are cultured on a nutrient medium, phenolic compounds get released heavily from the cut ends and their oxidation results in the browning of the plant tissues and culture media[11]. To overcome this problem, the explants were treated with a chilled antioxidant solution containing ascorbic acid, citric acid and PVP for 10-40 mins. The best combination to be found for this season was SST-5 (2% Tween-20 for 10 mins, 0.1% Bavistin for 8 mins, 0.1% $HgCl_2$ for 7 mins and 70% Alcohol for 30 secs) and AO-5 (75mg/l ascorbic acid, 35mg/l citric acid and 25mg/l PVPP for 40mins) showing the high CEF of 83.31±0.91% as shown in table 3.

For this season, the other nine surface sterilizing treatments were found to be not as suitable as SST-5 because for *T.*

arjuna, mercuric chloride is a better surface sterilising agent when compared to sodium hypochlorite (SST-1, SST-2, SST-3 and SST-4 contain sodium hypochlorite, hence they show less CEF). During 1st seasonal division, there was fresh sprouting on trees, which was very delicate; hence, explants were damaged by solid surface sterilizing treatments (SST-6, SST-7, SST-8, SST-9 and SST-10 are strong treatments).

But for controlling phenolic browning, mild treatments are not as effective as strong ones such as AO-5 because during this season, there was heavy leaching of phenolic compounds from cut ends; hence, the other antioxidants treatments viz. AO-1, AO-2, AO-3 and AO-4 are not suited well. Healthy explants, pre-treated with SST-5 and AO-5 after five weeks of culture are shown in figure 1. For comparison, explants showing high microbial contamination and phenolic exudation are also shown after five days of culture. Krishna et al[12] observed similar results to counter the problem of phenolic browning in the case of *Megnifera indica.* In case of *Terminalia catappa*, Phulwaria et al[21] found that ascorbic acid and citric acid can be potent agents to control phenolic browning. Shekhawat et al[23] also observed that treating the explants with antioxidant solutions promotes the better growth and development of shoots at the time of culture establishment.

Fig. 1: (a) Mother tree during the month of August (Seasonal division I), (b) Occurrence of phenolic browning and (c) microbial contamination in *Terminalia arjuna* after 5 days of culturing during seasonal division I, (d) and (e) healthy established nodal explants without any contamination and phenolic browning showing callus induction and shoot induction respectively after giving SST-5 and AO-5 treatments

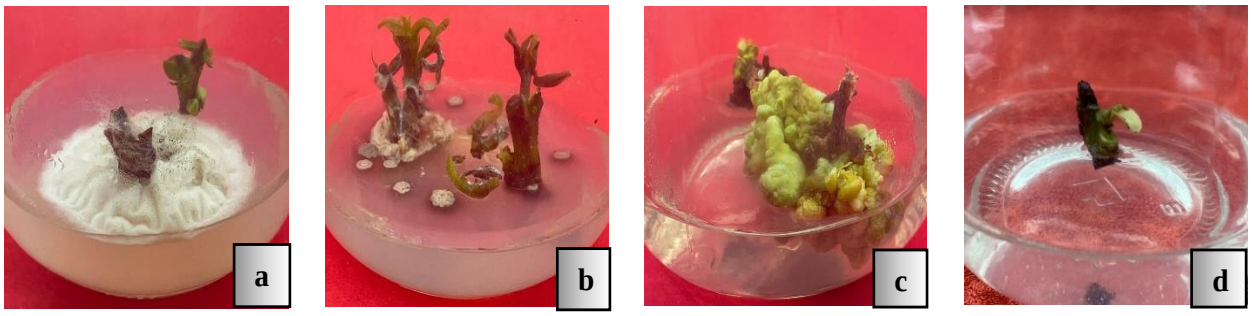

Fig. 2: Occurrence of microbial contamination in *Terminalia arjuna* after (a) 5 days of culturing, (b) 4 weeks of culturing as in seasonal division II there are prominent presence of recalcitrant endophytic microbes in plant tissues. (c) and (d) healthy established nodal explants without any contamination and phenolic browning showing callus induction and shoot induction respectively after giving SST-7 and AO-4 treatments.

Fig. 3: (a) Fresh sprouting on lopped mother tree during the month of November-December, (b) and (c) nodal explants collected from lopped tree showing high callus growth and shoot induction respectively

Fig. 4: Comparison of steps involved in surface sterilization of explants during different seasons

For seasonal division II (October to March): From November to April, the axillary and apical buds are in the dormant stage and therefore, they have very low levels of polyphenols when compared to active buds[24]. But in this seasonal division, high microbial contamination is prevalent because old tissues contain endophytic fungus or other

microbes, which cause heavy contamination after the culturing of the explant in nutrient media as these endophytic microbes are not killed by usual surface sterilizing protocols[20]. Hence, different pre-treatment protocols were carried out to remove microbial contamination and phenolic exudation. After that, it was observed that treating the explant with a mild anti-oxidant solution gave the best results in controlling phenolic exudation whereas strong treatments provided the best results in preventing microbial contamination.

Amongst the various treatments, microbial contamination was significantly controlled with SST-7 because SST-5 and SST-6 were mild treatments, hence were unable to control microbial contamination, which was prevalent in this seasonal division due to endophytic microbes present in aged tissues. The other three treatments (SST-8, SST-9 and SST-10) were very strong, damaging the explants and reducing the further CEF. The SST-7, with all eight AO treatments, resulted in significantly higher culture establishment frequency from 63.39±0.78% to 74.04±0.57%. The best combination was found when SST-7 (1% Tween-20 for 15mins,0.1% Bavistin for 12mins, 0.1% $HgCl_2$ for 8 min and 70% alcohol for 30secs) was combined with AO-4 (75 mg/l ascorbic acid, 35mg/l citric acid and 25mg/l PVPP for 30mins), resulting in a significantly higher culture establishment frequency of 74.04±0.57% because AO-4 is slightly mild as compared to other four antioxidant treatments (AO-1, AO-2, AO-3 and AO-5) which are strong hence have adverse effects on the explants (Fig. 4).

Effect of lopping of tree during seasonal division II: In the current research work, it was observed that the explants collected from unlopped mother tree showed lower bud break and higher contamination whereas the explants collected from lopped mother tree showed maximum bud break response and minimum contamination. Chaudhary et al[6] observed that the explants collected from lopped trees of Arjun responded better regarding percentage of bud break with minimum contamination. Phulwaria et al[20] recorded that the explants collected from aged trees had recalcitrant microorganisms that were very difficult to eliminate by surface sterilizing treatments.

Saha[22] also noted that explants collected from aged trees discharge more phenolic compounds in the culture media, reducing the micropropagation method's efficiency. When SST-5 was combined with all the eight antioxidant treatments, it was observed that the best combination was SST-5 (2% Tween-20 for 10 mins, 0.1% Bavistin for 8 mins, 0.1% $HgCl_2$ for 7 mins and 70% Alcohol for 30 secs) and AO-2 (100mg/l ascorbic acid, 50mg/l citric acid and 25mg/l PVPP for 30mins) which showed maximum CEF of 94.68±0.90% (Table 3).

For both the seasonal divisions, SST-3 and SST-4 showed the least CEF because both contain sodium hypochlorite as the main surface sterilizing agent, which was found to be the least effective as compared to mercuric chloride. Also, the high concentration of sodium hypochlorite (5% NaOCl) and more strong treatment of bavistin damaged the explants, further reducing the culture establishment frequency.

Conclusion

Particularly in woody plants like *Terminalia arjuna,* culture establishment is the first and the most difficult step for doing micropropagation, where various issues may arise. However, the current research work offers valuable information and methods to get around the problem of culture establishment. CEF of the explants collected from lopped trees was highest compared to those collected from seasonal divisions I and II. But when we compare both seasons without the effect of lopping, seasonal division I was the suitable period for explant collection compared to seasonal division II because in the seasonal division, trees I are sprouting.

Acknowledgement

Anita Rani acknowledges the University Grants Commission for providing her with a Junior Research Fellowship. We all are grateful to Department of Biotechnology (Chaudhary Devi Lal University, Sirsa) for cooperation, assistance and resources in completing this work.

References

1. Akhter S., Hossain M.I., Haque M.A., Shahriar M. and Bhuiyan M.A., Phytochemical screening, antibacterial, antioxidant and cytotoxic activity of the bark extract of *Terminalia arjuna*, *European Journal of Scientific Research*, **86(4)**, 543-552 **(2012)**

2. Amalraj A. and Gopi S., Medicinal properties of Terminalia arjuna (Roxb.) Wight & Arn.: a review, *Journal of Traditional and Complementary Medicine*, **7(1)**, 65-78 **(2017)**

3. Amente G. and Chimdessa E., Control of browning in plant tissue culture: A review, *J. Sci. Agric*, **5**, 67-71 **(2021)**

4. Chitbanchong W., Sardsud V., Whangchai K., Koslanund R. and Thobunluepop P., Control of rotting and browning of Longan fruit *cv. Biew Kiew* after harvested by sulphur dioxide treatment under various storage temperatures, *PJBS*, **12**, 1438-1447 **(2009)**

5. Choudhary M., Arya I.D. and Arya S., A complete review on Tissue Culture of *Terminalia arjuna*: A medicinally and economically important tree, *Biological Forum- An International Journal*, **4(1)**, 495-501 **(2022)**

6. Choudhary M., Arya I.D. and Arya S., Problems Encountered during *In vitro* Culture Establishment in *Terminalia arjuna*, *Annual Research & Review in Biology*, **35(12)**, 154-160 **(2020)**

7. Debergh P.C. and Zimmerman R.H., Micro propagation: Technology and Application, Kluwer, Dordrecht, 1-14 **(1991)**

8. Dwivedi S., *Terminalia arjuna* Wight & Arn. - A useful drug for cardiovascular disorders, *Journal of Ethnopharmacology*, **114(2)**, 114-129 **(2007)**

9. Gupta A.K., Harish, Rai M.K., Phulwaria M., Agarwal T. and Shekhawat N.S., *In vitro* propagation, encapsulation and genetic fidelity analysis of *Terminalia arjuna*: A cardioprotective medicinal tree, *Applied Biochemistry and Biotechnology*, **173(6)**, 1481-1494 **(2014)**

10. Habiba U., Reza S., Lal Saha M., Khan M.R. and Hadiuzzaman S., Endogenous bacterial contamination during *in vitro* culture of table banana: Identification and prevention, *Plant Tissue Cult. Biotechnol*, **12**, 117–124 **(2002)**

11. Jones A.M. and Saxena P.K., Inhibition of phenylpropanoid biosynthesis in *Artemisia annua L.*, a novel approach to reduce oxidative browning in plant tissue culture, *PloS One*, **8(10)**, e76802 **(2013)**

12. Krishna H., Sairam R.K., Singh S.K., Patel V.B., Sharma R.R., Grover M., Nain L. and Sachdev A., Mango explant browning: Effect of ontogenic age, mycorrhization and pre-treatments, *Scientia Horticulturae*, **118(2)**, 132-138 **(2008)**

13. Kumar G.P., Navya K., Ramya E.M., Venkataramana M., Anand T. and Kumar K.R.A., DNA damage protecting and free radical scavenging properties of *Terminalia arjuna* bark in PC-12 cells and plasmid DNA, *Free Radicals and Antioxidants*, **3**, 35-39 **(2013)**

14. Leelavathy S. and Sankar P.D., Curbing the Menace of Contamination in Plant Tissue Culture, *Journal of Pure & Applied Microbiology*, **10(3)**, 2145-2152 **(2016)**

15. Leifert C., Morris C.E. and Waites W.M., Ecology of microbial saprophytes and pathogens in tissue culture and field grown plants: reasons for contamination problem *in vitro*, *Crit. Rev. Plant Sci*., **13**, 139-183 **(1994)**

16. Mandal A., Das K. and Nandi D.K., *In vitro* bioactivity study of bark extract of *Terminalia arjuna* on probiotics, commercially available probiotic formulation, *International Journal of Phytopharmacology*, **1(2)**, 109–113 **(2010)**

17. Niedz R.P. and Bausher M.G., Control of in vitro contamination of explants from greenhouse-and field-grown trees, *In vitro Cell Dev. Biol. Plant*, **38**, 468-471 **(2002)**

18. Paarakh P.M., *Terminalia arjuna* (Roxb.) Wt. and Arn.: a review, *International Journal of Pharmacology*, **6(5)**, 515-534 **(2010)**

19. Permadi N., Nurzaman M., Alhasnawi A.N., Doni F. and Julaeha E., Managing Lethal Browning and Microbial Contamination in *Musa spp.* Tissue Culture: Synthesis and Perspectives, *Horticulturae*, **9(4)**, 453 **(2023)**

20. Phulwaria M., Ram K., Gahlot P. and Shekhawat N.S., Micropropagation of *Salvadora persica*- A tree of arid horticulture and forestry, *New Forests*, **42**, 317-327 **(2011)**

21. Phulwaria M., Ram K., Harish, Gupta A.K. and Shekhawat N.S., Micropropagation of mature *Terminalia catappa* (Indian Almond), a medicinally important forest tree, *Journal of Forest Research*, **17**, 202-207 **(2012)**

22. Saha D., Studies on factors influencing node culture establishment during *in vitro* shoot multiplication from mature *Schleichera oleosa (Lour.)* Oken tree, *Indian Journal of Natural Products and Resources*, **4(1)**, 102-109 **(2013)**

23. Shekhawat N.S., Rathore T.S., Singh R.P., Deora N.S. and Rao S.R., Factors affecting *in vitro* clonal propagation of *Prosopis cineraria*, *Plant Growth Regulation*, **12**, 273-280 **(1993)**

24. Siwach P. and Gill A.R., Optimization of conditions for culture establishment of nodal explants of *Ficus religiosa* L., *J. Appl. Biosci*., **39(2)**, 102-108 **(2013)**

25. Zhang X.R., Kaunda J.S., Zhu H.T., Wang D., Yang C.R. and Zhang Y.J., The genus *Terminala* (Combretaceae): An ethnopharmacological, phytochemical and pharmacological review, *Natural Products and Bioprospecting*, **9**, 357–392 **(2019)**.

(Received 21st July 2024, accepted 26th September 2024)