SYNERGISTICS EFFECT OF SILVER NITRATE AND ABSCISIC ACID IN CULTURE MEDIUM ON *IN VITRO* INITIATION AND MAINTEINANCE OF SOMATIC EMBRYOGENESIS IN PINUS ROXBURGHII

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Abstract

Low productivity of extrusion, initiation, maturation and germination of plantlets is an issue of concern in *Pinus roxburghii*. The focus of the current study was to study the effect of silver nitrate ($AgNO_3$) and abscisic acid (ABA) on extrusion, initiation, proliferation and maintenance of somatic embryogenesis (SE) in *P. roxburghii*. Cones of *P. roxburghii* were collected from the Botanical Garden, Institute of Botany, University of the Punjab, Lahore. Seeds were detached followed by inoculation on a modified LP (Loblolly Pine) medium having different levels of $AgNO_3$ (10, 20, 30 µM) and ABA (0.5, 1, 1.5 mg L^{-1}). The results illustrated that the co-addition of $AgNO_3$ and ABA increased the rate of extrusion and initiation compared to the solo application of $AgNO_3$ and ABA which demonstrated no significant outcomes. The rate of extrusion was highest using 30 µM $AgNO_3$+1.5 mg L^{-1}ABA (30.5 %), whereas increment in rate of initiation was observed from 1.02 % (10 µM $AgNO_3$+0.5 mg L^{-1}ABA) to 4.81 % (30 µM $AgNO_3$+1.5 mg L^{-1}ABA). On the contrary, modified LP medium supplemented with 6-Benzylaminopurine (1.995 µM), Kinetin (1.998 µM), 2,4-Dichlorophenoxyacetic acid (4.976 µM), ABA (4.91 µM) and $AgNO_3$ (10 µM) was used for proliferation and maintenance of SE of *P. roxburghii*. The results suggested that 30 µM of $AgNO_3$ along with 1.5 mg $L^{-1}$of ABA improved the extrusion and initiation results. To the best of our knowledge, the synergistic effect of $AgNO_3$ and ABA on the initiation and maintenance of SE in *P. roxburghii* has not been reported.

Keywords: Abscisic Acid, Initiation, Maintenance, Pinus, Silver Nitrate, Somatic Embryogenesis.

Introduction

Pinus roxburghii (commonly called chir pine) belongs to the family Pinaceae and has been well-known for its medicinal importance (Süntar *et al.,* 2012). The countries having chir pine populations are India, Bhutan, Jammu and Kashmir, Himachal Pradesh, Nepal, Sikkim, Uttarakhand, Pakistan, Afghanistan and the hills of western and eastern Himalayas (Arya *et al.,* 2000). Nasir *et al.* (1972) reported that amongst eight conifers species, two species (i.e., *Pinus brutia* and *Pinus halepensis*) are not in Pakistan. *P. roxburghii* has medicinal and economical importance for instance antiseptic, rubefacient, vermifuge and diuretic. It is also used to treat bladder and kidney disorders, eye, pharynx and ear diseases, liver diseases, hemoptysis, hemorrhages and sores, burn and wounds. Moreover, *P. roxburghii* is a cheaper source of fuelwood and its timber is economically significant for building and construction

purposes (Sharma *et al.*, 2015; Kanchan *et al.*, 2020).

Growth and performance of *P. roxburghii* are slow under normal environmental circumstances, while the growth rate can be improved via somatic embryogenesis. Somatic embryogenesis is the tissue culture practice in which immature or mature zygotic embryos initiate their development and eventually get matured, therefore, leading to plantlets (Malik *et al.*, 2020). SE has been reported by researchers in many gymnosperms such as *Pinus elliottii* (Yang *et al.*, 2020) *Pseudotsuga menziesii* (Lelu-Walter *et al.*, 2018), *Larix leptolepis* (Li *et al.*, 2018) and *P. pumila* (Tret'yakova and Shuvaev, 2015). The process of SE comprises four important steps i.e., initiation, maintenance, development and regeneration (Kong *et al.*, 2020). Grzyb and Mikuła (2019) reported that plants are distinctive in their capacity to produce somatic embryos. The rate of successful initiation is based on the type of explants and medium used for SE (Tripathi *et al.*, 2021). For most pine varieties, immature megagametophyte is used as initiation material for induction of SE, whereas, for others mature seed embryos can also be employed as the starting material (Arrillaga *et al.*, 2019). SE provides a suitable *in vitro* environment to study embryo development (Campos *et al.*, 2017) and the benefits of breeding programs can be attained via tree multiplication with increased wood quality, quantity and uniformity (Pullman and Bucalo, 2011). SE is also known to have enhanced secondary metabolite concentration under optimized PGR treatments (Khan et al., 2021).

The usage of chemicals like silver nitrate ($AgNO_3$) is vital for progression in SE. It is a stable, organic, non-hygroscopic and water-soluble compound. Barbasz *et al.* (2016) reported that $AgNO_3$ plays an important role in the initiation of SE, root development and shoot formation. Ethylene, a phytohormone, inhibits the plant growth via senescence (Khan *et al.*, 2014) and also influences the callus growth, shoot regeneration and hinders the initiation of SE (Purnhauser *et al.*, 1987). Malik *et al.* (2021) stated that an augmented level of $AgNO_3$ suppressed the production of ethylene, resulting in improving the initiation. Besides $AgNO_3$, ABA plays an important part in the initiation and maintenance of SE. High efficiency of maintenance of SE counts on the presence of ABA and sucrose. The importance of ABA in SE and embryo maturation has been reported by many researchers (Jimenez, 2005; Suzuki and McCarty, 2008; Wasilewska *et al.*, 2008). ABA is the important factor that determines the initiation, proliferation and maturation of somatic embryonal mass. ABA also helps in the accumulation of reserve materials for instance carbohydrates, lipids and proteins during the maturation phase

(Businge *et al.*, 2013). The objective of the current research was to study the effect of different doses of $AgNO_3$ along with ABA on extrusion and initiation of SE in *P. roxburghii*. The other aim was to practice the application of modified LP medium for the proliferation and maintenance of SE in *P. roxburghii*. To the best of our knowledge, the combined effect of $AgNO_3$ along with ABA on extrusion, initiation, proliferation and maintenance of SE in *P. roxburghii* has not been reported.

Materials and Methods

Collection and storage of female cones

Three Chir pine trees grown in Botanical Garden, Institute of Botany, University of the Punjab, Lahore were selected as the study trees. Trees having a large number of cones and good health were chosen for experimental purposes. Green colour female cones were detached from the trees followed by wrapping in brown paper. Cones were carried to the laboratory and stored at 4°C till further use.

Cutting of cone

For cutting, cones were positioned on the wooden board succeeded by cutting via hand cutting saw, whereas, for harder cones, an electric saw was used. The seeds were separated by using surgical tools such as forceps and scalpels etc. The wings were removed and the seeds were stored in a zip lock bag at 4°C.

Surface sterilization of seeds

Chir pine seeds were transferred in a clean conical flask and rinsed with running water for 10-15 minutes. After that, seeds were dipped in 10 % sodium hypochlorite solution succeeded by shaking in an orbital shaker for 5-10 minutes. Next, a few drops of 0.2% Tween-20 were added and placed on an orbital shaker for 10 minutes. After rinsing with water, seeds were treated with 20% hydrogen peroxide for 10 minutes and rinsed with autoclaved water under aseptic conditions.

LP medium preparation

For SE extrusion and initiation, LP medium was prepared as the detail provided in Table 1. Macronutrients, micronutrients, vitamins and iron-EDTA were added to the sterilized jar, whereas myo-inositol, maltose, casamino acid and $AgNO_3$ were added separately as shown in Table 1. Activated charcoal and Gelrite were mixed after pH adjustment to 5.7. LP medium having different concentrations of $AgNO_3$ (10, 20 and 30 µM L^{-1}) along with ABA (0.5, 1 and 1.5 mg L^{-1}) was used for extrusion and initiation.

On the contrary, for proliferation and maintenance of initiated embryonal mass, a modified LP medium was used. The detailed

components of proliferation and maintenance medium are illustrated in Table 2.

The medium was shifted into 1000 mL bottle followed by autoclaving for 20 minutes at pressure and temperature of 15 lbs $inch^{-2}$ and 121°C. After sterilization, LP medium was supplemented with ABA and L-Glutamine (filter-sterilized). Next, LP medium was poured into sterilized petri plates under aseptic conditions and allowed to cool at room temperature.

Seed inoculation

For inoculation, seeds were opened by using septic forceps. The seeds coat, nucellus and integument were detached and megagametophyte was cautiously placed on different concentrations of LP media. Each Petri plate was inoculated with three seeds and sealed with parafilm. The plates were stored at 23-25°C in the dark and cultures were examined regularly.

Extrusion and initiation of SE

Extrusion is the phase when the zygotic embryo was forced out at the micropylar end, whereas initiation leads to continual growth and production of enough embryonal tissues. Extrusion and initiation % were calculated independently.

Proliferation and maintenance of SE

The initiated embryonal tissue separated from megagametophyte was placed on the LP modified medium for proliferation of SE as demonstrated in Table 2. The subculturing of embryonal mass was carried out after every 3-4 weeks till 5-20 mg per culture was obtained.

Statistical analysis

Data obtained from the experiments were analyzed via SPSS (version 22). Analysis of variance (ANOVA) was carried out to determine the mean at a statistical significance level of 5%. The mean values were compared by Duncan's multiple range test (DMRT) at a probability level of 0.05. All the experiments were performed in triplicate.

Results and Discussion

In the present study, an immature zygotic embryo was cultured on LP medium followed by the analysis of various SM stages such as extrusion, initiation, proliferation and maintenance.

Extrusion

The extrusion of embryonal tissue from the micropylar end of the megagametophyte is termed as early extrusion, whereas progressive development results in late extrusion. In the current study, the synergistic effect of $AgNO_3$ and ABA on SE of *P. roxburghii* was observed. The results illustrated that % extrusion was increased correspondingly with increment in $AgNO_3$ and ABA concentration. A significant increase in % extrusion was

observed by supplementing the LP medium with 30 µM $AgNO_3$ and 1.5 mg L^{-1} ABA in tree 1 and tree 2 compared to tree 3 (Figure 1). Pinto *et al.* (2008) stated that extrusion and initiation of *P. elliottii* depended on the inoculating medium, the concentration of plant growth promoters, explant type and zygotic embryo stage. Somatic embryogenesis in members of Pinaceae has been reported by many researchers such as *P. pinea* (Carneros *et al.,* 2009), *P. halepensis* (Montalbán *et al.,* 2013) and *P. caribaea* (Nunes *et al.,* 2018).

In the present study, an immature zygotic embryo was used for the extrusion in *P. roburghii*. Usage of mature and immature zygotic embryo as an inoculant for SE has been reported by many researchers (Satish *et al.,* 2016; Maruyama and Hosoi, 2019). It was noted that the extrusion occurred within 1 to 3 weeks of initial culture. The different phases of extrusion are illustrated in Figure 2. Pullman (2018) stated that extruded zygotic embryos have the same appearance as somatic embryos and cannot easily be distinguished except by observations of continued growth.

The current findings illustrated that difference in % extrusion was significant between media varying in the concentration of $AgNO_3$ and ABA i.e., extrusion % was increased from 2.98 to 30.5% as the concentration increased from 10 µM $AgNO_3$+0.5 mg L^{-1} ABA to 30 µM $AgNO_3$+1.5 mg L^{-1} ABA (Figure 1). Yang *et al*. (2020) reported that addition of 1-2 mg L^{-1} of ABA improved the extrusion and initiation in *P. ellottii*. ABA plays a key part in maintaining the metabolic and physiological activities of the female gametophyte and embryonal mass (Yang *et al.,* 2020). In the current study, maximum extrusion was observed in the cones collected in July and August over other months collected cones which showed a reduction in the rate of extrusion. The results illustrated that maximum extrusion rate was noted in tree 2 (33.9%) followed by tree 1 (32.5%), whereas the lowest extrusion rate was observed for tree 3 (1.01%). Similar to our findings, Maruyama *et al.* (2005) reported 2.4% improvement in extrusion % in *P. thunbergia* inoculated on embryogenic medium supplemented with 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). The order of % extrusion displayed by inoculation of an immature zygotic embryo on LP medium supplemented with different doses of $AgNO_3$ andABA was tree 2 > tree 1 > tree 3.

Initiation

Our findings illustrated that LP medium having different levels of $AgNO_3$ along with ABA showed improvement in initiation rate. The maximum initiation was observed by the addition of 30 µM $AgNO_3$ and 1.5 mg L^{-1} ABA, whereas minimum % initiation was exhibited by the application of 10 µM $AgNO_3$ and 0.5 mg L^{-1} ABA. *Pullman et al.* (2016) stated that the success of initiation depends on the plant family and zygotic embryo stage. Analogous to our findings, Pullman *et al.* (2003) reported that addition of 20 µM of $AgNO_3$ in an embryogenic medium improved initiation in *P. taeda* compared to control having no $AgNO_3$. They also described that co-application of $AgNO_3$ and auxin or ABA improved initiation and regeneration in *P. taeda* over solo application of $AgNO_3$. The highest initiation % was noted in July compared to other months such as % initiation on July 14 and 29 was 4.57 and 4.7 %, whereas % initiation was 4.81% on 13 August collected cones (Figure 1). The results demonstrated that all the extrusions did not show initiation i.e., out of 7.02% extrusion , only 1.3% cultures showed initiation. Different phases of initiation are illustrated in Figure 3.

Out of three studied chir pine trees, considerable improvement in initiation was observed in tree 2 followed by tree 1, whereas tree 3 did not show any initiation. The possible reason for improved initiation may be attributed to the high level of vitamins, organic acids and sugars in inoculating medium (De Silva *et al.,* 2015). Thus, the order of initiation % was tree 2 > tree1 > tree 3. In line with our findings, Li and Huang (1996) reported that the

addition of 11.1 mM of myo-inositol and 29.4 mM of $AgNO_3$ enhanced the SE starting from extrusion to proliferation. In the current study, immature zygotic embryo of *P. roxburghii* were used to obtain the initiated embryonal mass. Analogous to our findings, Pullman *et al.* (2016) reported significant improvement in initiation rate by using immature seeds compared to mature seed. Wu *et al.* (2012) asserted that inoculation of mature seeds on embryonal medium decreased the initiation rate over immature seeds and it may be due to the leaching of my-inositol.

In the current study, improvement in initiation was observed in LP medium supplemented with different doses of $AgNO_3$ and ABA. Pullman *et al.* (2003) reported that embryogenic medium having ABA improved the initiation and proliferation. In the current research, 0.5-1.5 mg L^{-1} concentration of ABA along with different levels of $AgNO_3$ improved the initiation in *P. roxburghii*. Analogous to our findings, Lema- Rumińska *et al.* (2013) reported that the application of 1

µM level of ABA stimulated the initiation, whereas further augmented level of ABA ranging from 10 to 100 µM inhibited the initiation rate.

Proliferation and Maintenance

The embryonal tissues showing the initiation were further inoculated on LP modified medium containing 2,4-D (4.976 µM), Kinetin (1.998 µM), BAP (1.995 µM), $AgNO_3$ (10 µM) and ABA (4.91 µM). Similar to our findings, Dadjo *et al.* (2015) reported that Murashige and Skoog (MS) medium supplemented with 8.45 mg L^{-1} of $AgNO_3$ improved the induction, proliferation and regeneration in SE in *vitix doniana* compared to MS medium supplemented with 16.9 and 25.35 mg L^{-1} concentration of $AgNO_3$. In the current study, a lower concentration of $AgNO_3$ (i.e., 10 µM) was used and seemed to be effective for the proliferation and maintenance of SE in *P. roxburghii*. It has been reported by many researchers that supplementation of embryogenic medium with $AgNO_3$ improved the SE in species for instance *Hedychiurn bousigonianum* (Sakhanokho *et al.,* 2009), *Eleusine coracana* (Kothari-Chajer *et al.*, 2008) and *Paspalum scrobiculatum* (Vikrant and Rashid, 2002). The results illustrated that few cultures lost their embryonal potential during maintenance, whereas the appearance of a dome-shaped head of embryonal mass illustrated the progression of SE in the combined application of $AgNO_3$ and ABA (Figure 4).

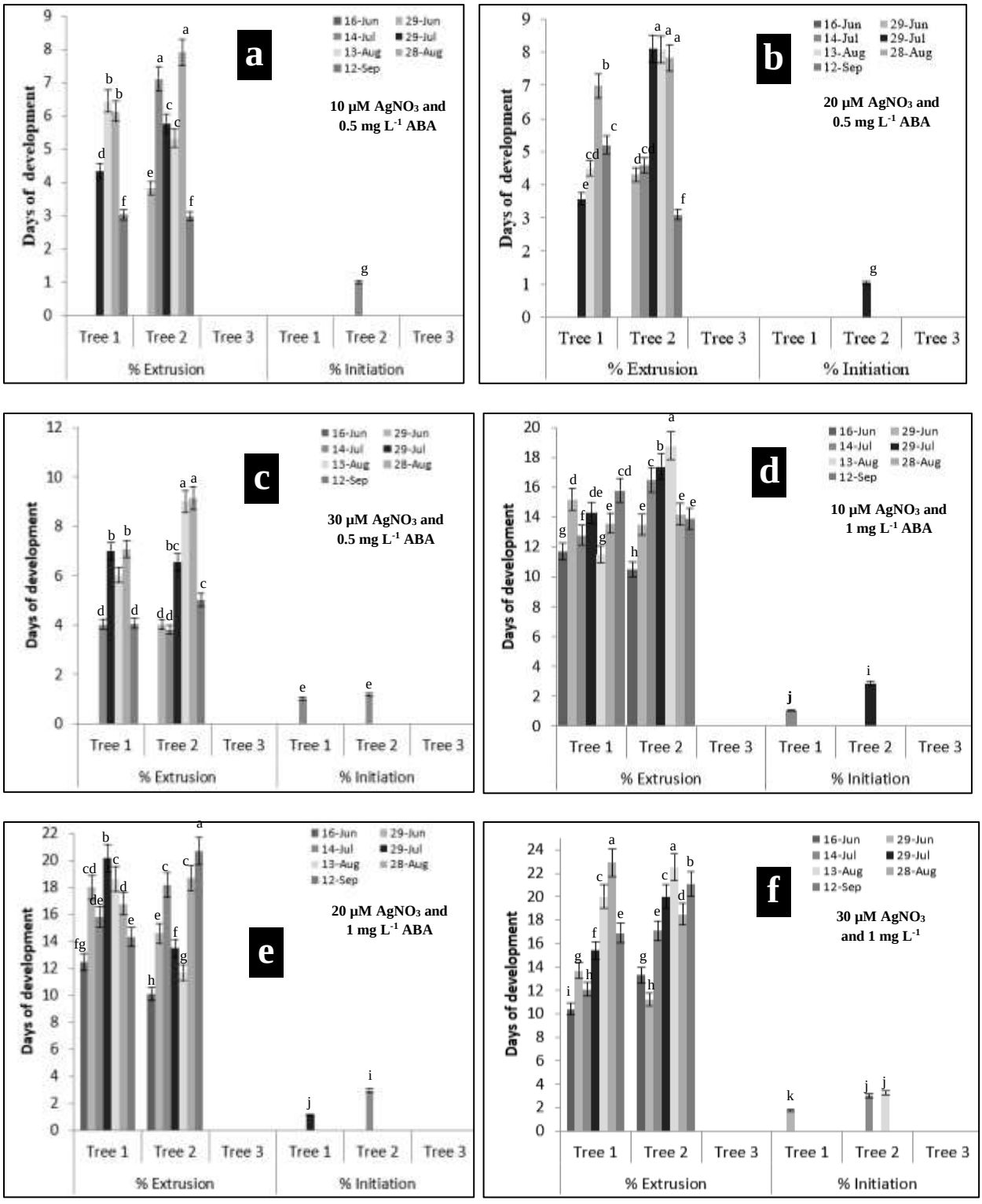
In recent studies, LP medium having auxin, cytokinin and ABA improved the proliferation and maintenance. Krajňáková *et al.* (2009) stated that the addition of plant growth regulators such as cytokinins and ABA in embryogenic medium improved the initiation and maintenance of SE.

Conclusion

The current study concluded that synergistic effect of $AgNO_3$ and ABA improved the rate of extrusion and initiation of SE in *P. roxburghii*. The results illustrated that co-application of 30 µM $AgNO_3$ and 1.5 mg L^{-1} ABA improved the extrusion and initiation rate over other doses. It was also observed that all the extrusions did not lead to initiation. On the contrary, a modified LP medium having 10 µM of $AgNO_3$ and 4.91 µM of ABA seemed to improved the proliferation and maintenance in *P. roxburghii*. Our results give the basis for a more efficient SE with modified LP media containing other chemical compounds or growth regulators in the future.

Conflict of interest

The authors declare no competing interests.


a
10 µM AgNO3 and 0.5 mg L-1 ABA
b
20 µM AgNO3 and 0.5 mg L-1 ABA
c
30 µM AgNO3 and 0.5 mg L-1 ABA
d
10 µM AgNO3 and 1 mg L-1 ABA
e
20 µM AgNO3 and 1 mg L-1 ABA
f
30 µM AgNO3 and 1 mg L-1
16-Jun
29-Jun
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13-Aug
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12-Sep
Days of development
Tree 1
Tree 2
Tree 3
% Extrusion
% Initiation

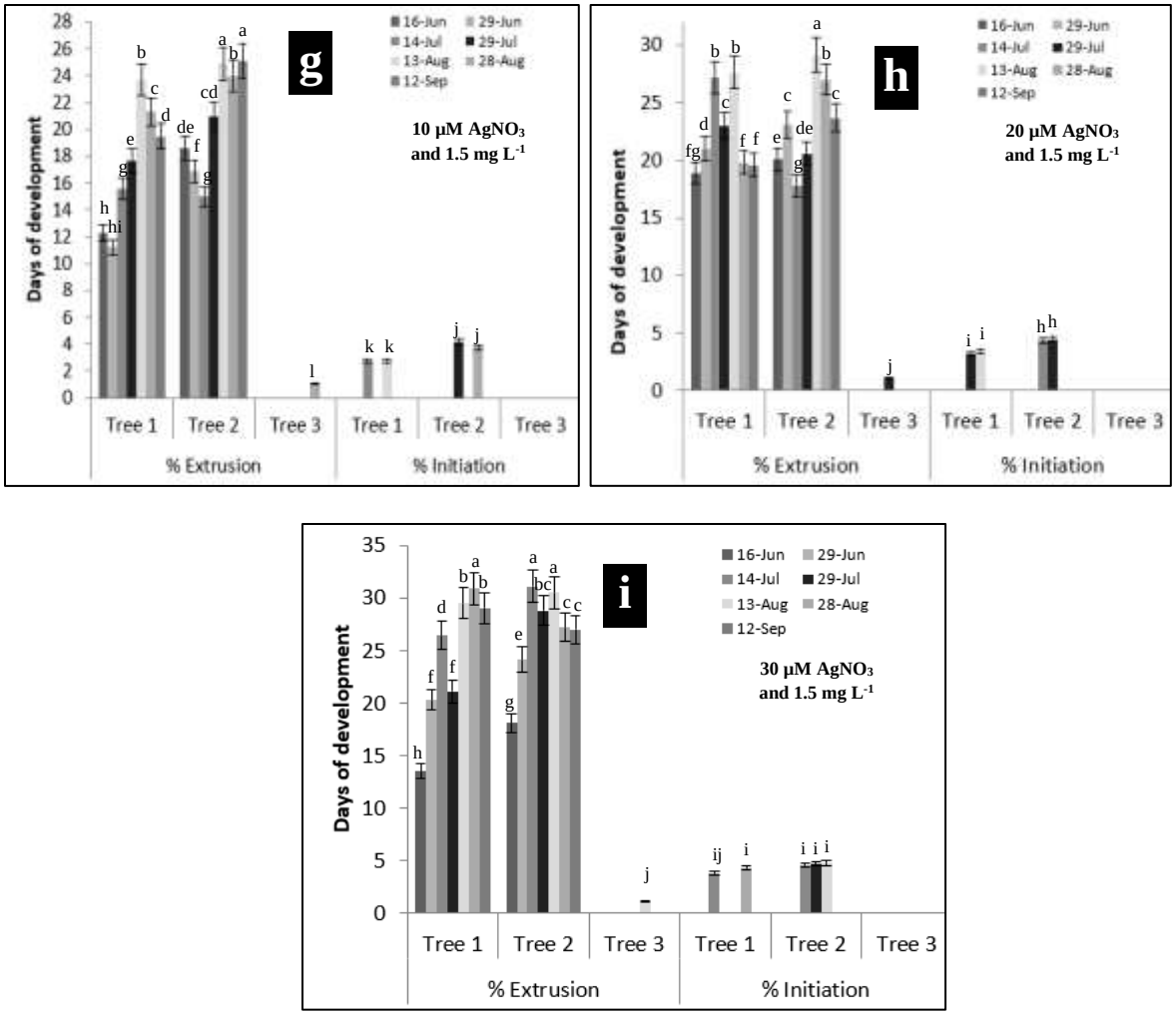

Figure 1(a-i): Effect of LP medium having different concentrations of $AgNO_3$ and ABA on extrusion and initiation in three different tree of *P. roxburghii*. Different letters indicate significant differences among treatments ($p \leq 0.05$)

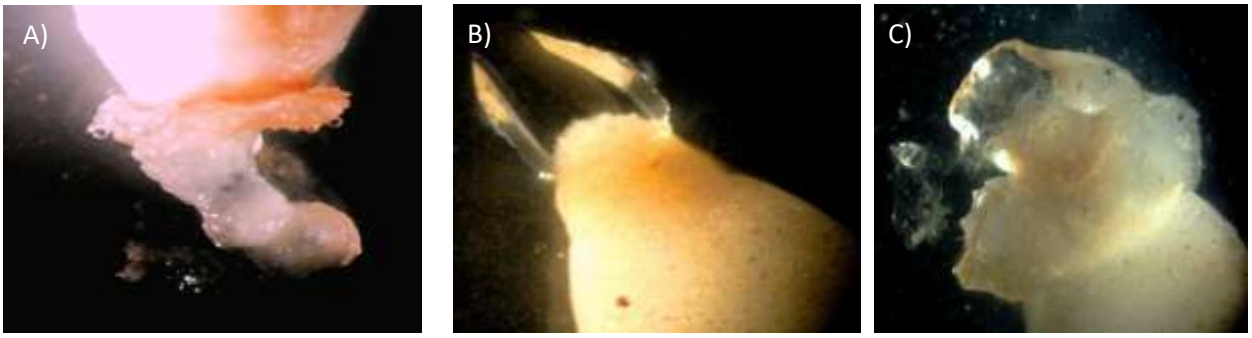

Figure 2: showing different extrusion stages on LP medium supplemented with $AgNO_3$ along with ABA during SE of *P. roxburghii*. (A) somatic embryos came out from the micropylar end as curved shaped structure, (B) Two straps extruded out from the micropylar end of megagametophyte and (C) Embryonal tissue came out and began to spread in all directions.

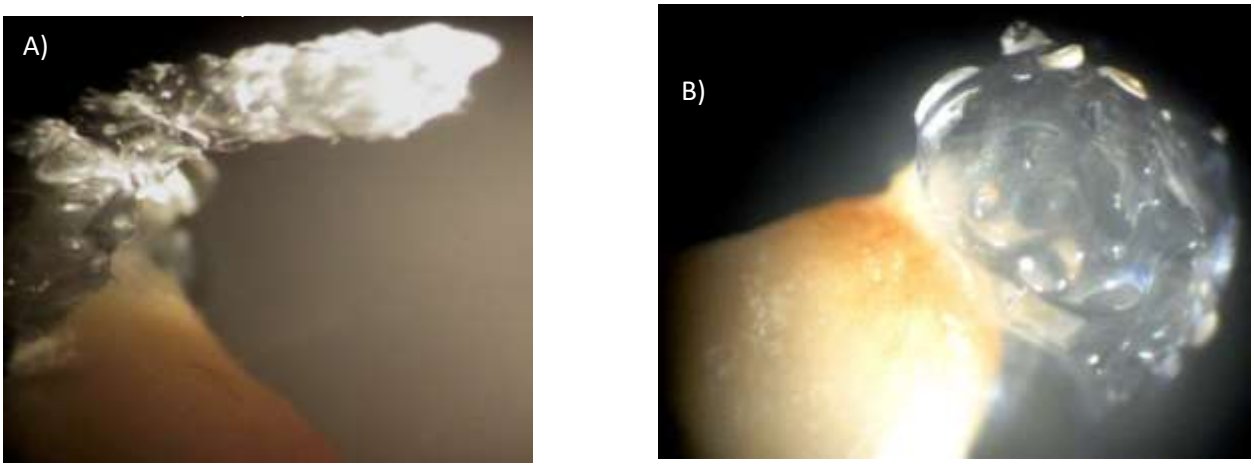

Figure 3: Initiation of SE by supplementing the LP medium with $AgNO_3$ along with ABA during SE of *P. roxburghii*., (A) spiky head of somatic embryos were examined under the stereomicroscope and (B) Initiation of embryonal tissue started but stopped to grow further.

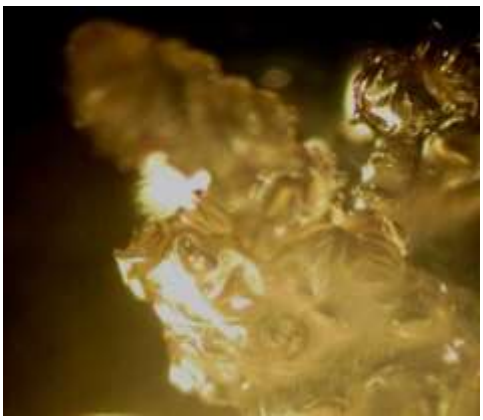

Figure 4: Maintenance of SE recorded on modified LP medium during SE of *P. roxburghii*. Dome shaped head of proliferating mass of embryonal tissue emerged on maintenance medium.

Table 1: Formulation of LP (loblolly pine) extrusion and initiation medium

Components	**Stock Concentration**	**LP Medium Final Concentrations**
Macronutrient	**mgL^{-1}(10×)**	**mgL^{-1}**
NH_4NO_3	2000	200
KNO_3	9099	909.9
KH_2PO_4	1361	136.1
$Ca(NO_3)_2.4H_2O$	3000	300
$MgSO_4.7H_20$	3000	300
$MgCl_2.6H2O$	1017	101.7
Micronutrient	**mgL^{-1} (50×)**	**mgL^{-1}**
KI	207.5	4.15
H_3BO_3	775	15.5
$MnSO_4.H_2O$	525	10.5
$ZnSO_4.7H2O$	733.4	14.668
$Na_2MoO_4.2H_2O$	6.25	0.125
$CuSO_4.5H_2O$	8.625	0.1725
$CoCl_2.6H_2O$	6.25	0.125
Iron-EDTA	**mgL^{-1} (100×)**	**mgL^{-1}**
$FeSO_4.7H_2O$	1390	13.9
Na_2EDTA	1865	18.65
Vitamins	**mgL^{-1} (50×)**	**mgL^{-1}**
Thiamine HCl	50	1.0
Pyridoxine HCl	25	0.5
Nicotinic acid	25	0.5
Glycine	100	2.0
Growth Regulator	**mgL^{-1}**	
1-Naphthaleneacetic acid (NAA)	2.0	
6-Benzylaminopurine (BAP)	0.55	
Kinetin	0.53	
Others	**mgL^{-1}**	
L-Glutamine	450	
Maltose	15,000	
Myo-inositol	20,000	
Casamino acid	500	
Gelrite	2,000	
pH	5.7	

Table 2: Formulation of modified LP maintenance medium

Components	**Stock Concentration**	**LP Medium Final Concentrations**
Macronutrient	**mgL^{-1}(10×)**	**mgL^{-1}**
NH_4NO_3	6038	603.8
KNO_3	9099	909.9
KH_2PO_4	1361	136.1
$Ca(NO_3)_2.4H_2O$	2362	236.2
$MgSO_4.7H_20$	2465	246.5
$MgCl_2.6H2O$	1017	101.7
$Mg(NO_3)_2.6H_2O$	2565	256.5
Micronutrient	**mgL^{-1} (50×)**	**mgL^{-1}**
207.5	207.5	4.15
H_3BO_3	775	15.5
$MnSO_4.H_2O$	525	10.5
$ZnSO_4.7H2O$	733.4	14.6
$Na_2MoO_4.2H_2O$	6.25	0.125
$CuSO_4.5H_2O$	8.625	0.1725
$CoCl_2.6H_2O$	6.25	0.125
Iron-EDTA	**mgL^{-1} (100×)**	**mgL^{-1}**
$FeSO_4.7H_2O$	6.95×100=695	6.95
Na_2EDTA	933	9.33
Vitamins	**mgL^{-1} (50×)**	**mgL^{-1}**
Thiamine HCl	1.0×50=50	1.0
Pyridoxine HCl	25	0.5
Nicotinic acid	25	0.5
Glycine	100	2.0
Growth Regulator	**mgL^{-1}**	
BAP	0.45	
Kinetin	0.43	
ABA	1.3	
*2,4-Dichlorophenoxyacetic acid (*2,4-D)	1.1	
Others	**mgL^{-1}**	
$AgNO_3$	10 µM	
L-Glutamine	450	
Sucrose	30,000	
Myo-inositol	1,000	
Casamino acid	500	
Gelrite	2500	
pH	5.7	

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