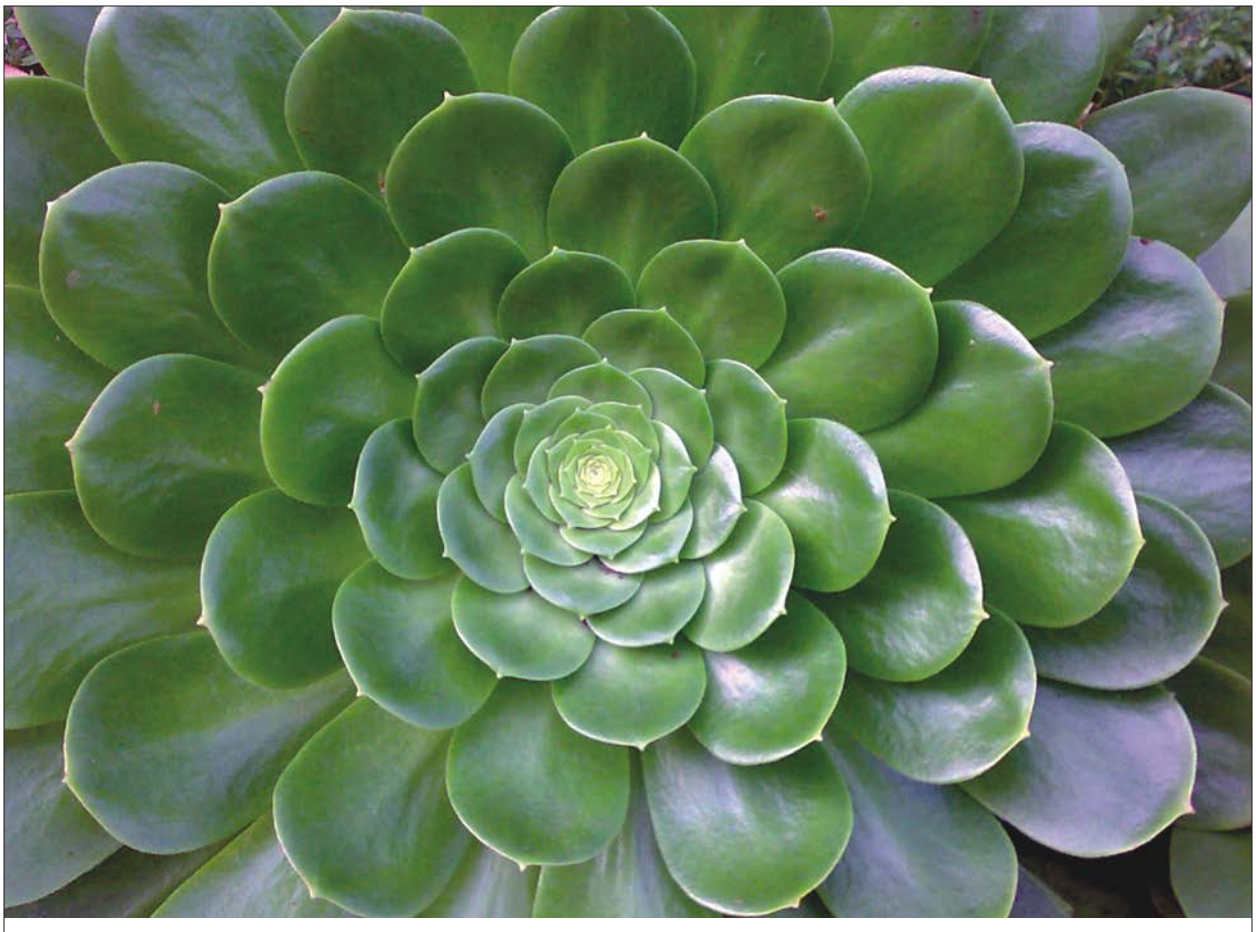

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# Smoke-saturated Water Influences Somatic Embryogenesis Using Vegetative Shoot Apices of Mature Trees of *Pinus wallichiana* A.B. Jacks

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**Abstract:** This study highlights for the first time the influence of smoke-saturated-water on somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana*, an important pine tree of Himalayan region. The addition of smoke-saturated -water derived from the local grasses has a significant effect on the different developmental stages of somatic embryogenesis of *P. wallichiana*. Smoke-saturated-water at a concentration of 10% in the DCR basal medium (Gupta and Durzan, 1985) resulted in the higher percentage of embryogenesis in all the three genotypes of *P. wallichiana* as compared against control. Lower concentrations of smoke-saturated water do not have any effect on somatic embryogenesis. On the other hand higher concentrations of smoke-saturated water inhibited embryogenesis in all the three genotypes of *P. wallichiana*. The presence of smoke-saturated-water in the maturation medium increased the rate of embryo development as evidenced by the occurrence of more number of well matured somatic embryos (PW-37; PW-34; PW120-39) recovered per gram fresh-weight of embryogenic tissue as compared against control. Maximum number of somatic embryos germinated successfully and resulted in the formation of vigorous seedlings compared against control. These observations suggest that the active ingredient (s) in smoke-saturated-water play a regulatory role in plant development. This plays an important role in commercial forestry.

**Key words:** Apical shoot, fire, grass, Himalayan blue pine, smoke, somatic embryogenesis

## Introduction

Plant-derived smoke extracts are known to stimulate seed germination in a number of species (Brown, 1993; Baxter *et al*., 1994; Dixon *et al*., 1995; Brown *et al*., 2003; Brown and Botha, 2004; Keeley and Fotheringham, 1998; Light *et al*., 2002; Light and van Staden, 2004). Fire is well established as a major evolutionary driving force in seed biology and is clearly regulated and mediated *via* both physical and chemical cues involved in the germination process (Van Staden *et al*., 2000, 2004). Smoke released from burning vegetation contains a chemical signal that triggers germination of species from different parts of the world (Baxter *et al*., 1994; Baxter and van Staden, 1994; Drewes *et al*., 1995; Thomas and Van Staden, 1995). It is used in horticulture to stimulate seed germination of vegetable crops, such as lettuce and celery (Drewes *et al*., 1995; Thomas and van Staden, 1995). Recently a highly active, heat stable, long lasting compound, 3-methyl-2H-furo (2, 3-c) pyran-2-1, that stimulates seed germination was isolated from plant-derived smoke-water using bioactivity-guided-fractionation. This compound is water soluble, heat stable and can be stored for long periods as an aqueous solution. It also retains its activity after autoclaving and long periods of storage (Van Staden *et al*., 2000, 2004). Flematti *et al*. (2004) has also reported the same compound, isolated from smoke produced by burning cellulose. Responses to smoke other than the breaking of seed dormancy have also been observed. Smoke stimulated flowering in *Cyrtanthus ventricosus* (Keeley, 1993), whereas

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smoke solutions stimulated root initiation and development in the hypocotyls of mung beans (Taylor and Van Staden, 1996) and promoted somatic embryogenesis in geranium (*Pelargonium hortorum* Bailey) (Senaratna *et al*., 1999).

*Pinus wallichiana* AB. Jacks (Himalayan Blue pine or Bhutan pine) is a native of the outer Himalayas and prevalent in the Northern Himalayan range. It is also found in the principle valleys of the Himalayas from Kashmir to Bhutan and extending up to Arunachal Pradesh in north-eastern India. Between 2000 to 3500 m above sea level, Himalayan blue pine or Bhutan pine is an important indigenous pine species in India, Bhutan and Nepal. Due to ever-increasing population, natural forests of blue pine and associated trees of the region are being cleared for cultivation at an alarming rate. Very few trees are also available in the Western Ghat Forest as result of shifting cultivation trial programme by Forestry Department for the conservation of important forestry species (Kumar, 1975; Saldanha, 1985). It provides valuable natural resources that contribute significantly to the local economy of the country and stabilizes the land that supplies water to the millions of people living in the Himalayan river basins. Somatic embryogenesis is the most promising technology to multiply high-value forest trees and is expected to play an important role in increasing productivity, sustainability and uniformity of future forests throughout the world (Malabadi and van Staden, 2005a-d). Unfortunately, sexual production of trees yields trees with variable characteristics. Methods to propagate large numbers of genetically superior conifer trees are needed. Cloning of superior genotypes can produce uniform plantlets with elite characteristics from clearly defined parents (Malabadi and Nataraja, 2006). However, very few successful reports of cloning of adult trees in conifers are available (Bonga and Von Aderkas, 1993; Ruaud, 1993; Ruaud *et al*., 1992; Bonga and Pond, 1991; Bonga, 1996, 2004; Westcot, 1994; Smith, 1997; Malabadi *et al*., 2003; Malabadi *et al*., 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006).

This study highlights the effects of smoke water in the process of somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* AB. Jacks. Somatic embryogenesis is the phenomenon of embryo development in response to chemical stimuli. Somatic embryos arise from somatic cells without genetic recombination. Lower percentage of *in vitro* plantlet regeneration of *Pinus wallichiana* was achieved by organogenesis of mature zygotic embryos (Konar and Singh, 1980; Bastola *et al*., 1991; Mathur and Nadagauda, 1998, 1999). No reports are available on somatic embryogenesis of Himalayan blue pine. This has created a considerable interest to evaluate the influence of plant-derived smoke solutions on somatic embryogenesis in *P. wallichiana*. Present results confirmed that Smoke-Saturated Water (SSW) influences somatic embryogenesis in *P. wallichiana* and can be used as plant growth hormone in other conifers.

## Materials and Methods

*Preparation of Smoke-Saturated-Water*

The Smoke-saturated Water (SSW) was prepared according to the procedure described by Thomas and van Staden (1995) and Dixon *et al*. (1995). This was achieved by slow burning of a mixture of two local semi-dry grasses *Aristida setacea* and *Cymbopogon martini* (Graminiaceae). The resulted smoke was first passed into a metal drum connected to a flask containing 500 mL of distilled water through a pipe. The smoke was forced to pass through the water by blowing the air by a fan or compressed air. The smoke-saturated-water was collected and stored at 2°C until further use. The different concentrations of smoke -saturated- water (10, 15 and 20 %) were used in the following experiments.

*Plant Material and Initiation of Embryogenic Cultures*

Apical shoots (Fig. 1A) from mature trees (13 years old) of *Pinus wallichiana* AB. Jacks of 3 genotypes (PW10, PW39 and PW120) were collected from the Western Ghat Forests, India ($14^0$ 5'

to 15°25' N latitude and 74°45' to 76°E longitude with an average rainfall of 85 cm.). Apical shoots were harvested during the month of May. They were cleansed with 1% Citramide (Sodium hypochlorite 3.5%) for 5 min and rinsed thoroughly with sterilized distilled water. They were then surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4-times with sterile double distilled water. Transverse-thin sections, approximately 0.5-1.0 mm thick, were cut using a sharp sterilized blade or a scalpel from apical shoots (upper part with 2 to 3 sections only) for the initiation of embryogenic tissue. These shoot apical shoot sections were cultured individually on full strength inorganic salts DCR (Gupta and Durzan, 1985) basal medium containing 0.2 $gL^{-1}$ polyvinyl pyrollidine (PVP) (Sigma), 2 g $L^{-1}$ Gellan gum (Sigma), 30 g $L^{-1}$ maltose (Analar, Sigma) and 0.3% activated charcoal (Sigma) without growth regulators (Pre-culture medium I). For the smoke-saturated-water treatment, preculture medium was incorporated with different concentrations of smoke-saturated water (10, 15 and 20%). The preculture medium

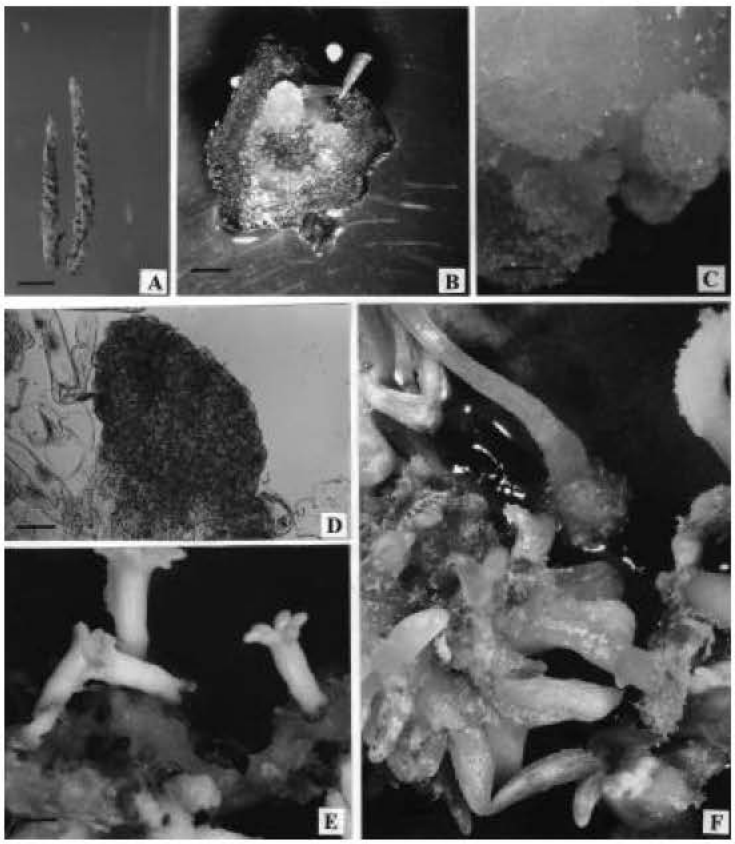


Fig. 1: Influence of smoke-saturated water on establishment of embryogenic tissue from vegetative shoot apices of mature trees of *Pinus wallichiana* A. B. Jacks. (a) Apical shoots harvested from mature trees (outer scales removed) (scale bar 1 cm = 2.5 cm). (b) Cold-pretreated apical shoot section showing the initiation of white mucilaginous tissue on initiation medium containing 10% of SSW (scale bar 1 cm = 2.5 cm). (c) Proliferation of embryogenic tissue on maintenance medium containing 10% SSW (scale bar = 1 cm). (d) Cells with cleavage polyembryony showing the sign of head formation (bar = 1.2 cm). (e) Well matured somatic embryos showing an advanced cotyledonary stage ready for the germination (bar = 0.9 cm). (f) Germination of somatic embryos on a medium containing 10% SSW (scale bar = 0.4 cm)

without smoke-saturated water is served as a control. The cultures were raised in 25×145 mm glass culture tubes (Borosil) containing 15 mL of medium. These cultures were incubated in dark at 2°C for 3 days. Thin apical shoot sections after incubation in dark at 2°C for 3 days were subcultured on full strength DCR basal medium. For the initiation of embryogenic tissue, full strength DCR basal medium was incorporated with 0.2 g $L^{-1}$ PVP, 2 g $L^{-1}$ Gellan gum, 1 g $L^{-1}$ L-glutamine, 1 g $L^{-1}$ casein hydrosylate, 1 g $L^{-1}$ meso-inositol (Sigma) and supplemented with 22.62 μM 2, 4-D, 26.85 μM NAA and 8.87 μM BA (Sigma) was used as an initiation medium (II) (Malabadi *et al*., 2003; Malabadi *et al*., 2004a, b; Malabadi and Van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006). Effect of smoke-saturated-water was also studied on the initiation of embryogenic tissue by incorporating the different concentrations of Smoke-Saturated-Water (SSW) (10, 15 and 20%) in the initiation medium. Initiation medium without smoke-saturated-water is considered as control. The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium was then sterilized by autoclaving at 121°C and 1.05 kg $cm^{-2}$ for 15 min. L-glutamine and casein hydrolysate were filter sterilized and added to the medium after it had cooled to below 50°C. All the cultures were maintained in the dark at 25±2°C.

*Maintenance of Embryogenic Cultures*

The embryogenic tissue producing proembryonal masses was again subcultured onto maintenance medium (Fig. 1C). The full-strength DCR basal medium containing 60 g $L^{-1}$ maltose, 4 g $L^{-1}$ Gellan gum supplemented with 2.26 μM 2,4-D, 2.68 μM NAA and 0.88 μM BA (Maintenance medium) (III) and different concentrations of smoke-saturated water (SSW) (10, 15 and 20%) was used for this purpose. On the maintenance medium, embryonal suspensor masses were cultured for 30 days with 2 subcultures. The presence of embryonal masses was determined by microscopic observations (Fig. 1D) (Malabadi *et al*., 2003, 2004a, b; Malabadi and Van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006).

*Maturation of Somatic Embryos*

After partial desiccation of 24 h (Malabadi and Van Staden, 2005a-d; Malabadi and Nataraja, 2006) the embryogenic tissue was transferred to maturation medium to induce cotyledonary embryo development (Fig. E). The full strength DCR basal medium with 60 g $L^{-1}$ maltose, 37.84 μM ABA and 5 g $L^{-1}$ Gellan gum (IV) containing various concentrations of smoke-saturated-water (10, 15 and 20%) was tested for this purpose. All the cultures were placed in the dark at 25±2°C and these maintained for 8 to 12 weeks (Malabadi *et al*., 2003, 2004a, b; Malabadi and Van Staden, 2003, 2005a-d, 2006; Malabadi and Nataraja, 2006).

*Germination and Plantlet Recovery.*

After 6 to 8 weeks of maturation in presence of ABA and higher concentrations of maltose, advanced cotyledonary somatic embryos were picked from the cultures for germination. The germination medium (V) used was half DCR basal medium with 2 g $L^{-1}$ Gellan gum containing various concentrations of smoke-saturated-water (SSW) (10, 15 and 20%). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite. Plantlets were placed in growth room under a 16 h photoperiod (50 μmol $m^{-2}$ $sec^{-1}$) for hardening (Malabadi *et al*., 2003, 2004a, b; Malabadi and van Staden, 2003, 2004, 2005a-d, 2006; Malabadi and Nataraja, 2006).

*Statistical Analysis*

In all the above experiments each culture tube received a single explant. DCR basal medium without smoke-saturated-water is served as a control (In case of control also 100 thin sections were

cultured for one set of experiment for each genotype). Each replicate contained 25 cultures and one set of experiment is made up of 4 replicates (100 transverse-thin - sections of vegetative shoot apices were cultured for each genotype for one set of experiment). All the experiments were repeated 4 times (total 400 cultures). Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA and the differences contrasted using a Duncan's multiple range test. All statistical analysis was performed at the 5% level using the SPSS statistical software package.

## Results and Discussion

On the basis of results, it is confirmed that apical shoots of three genotypes of *P. wallichiana* collected during May were able to produce embryogenic tissue on the initiation medium (Fig. 1A and B). On the other hand, explants produced non-embryogenic tissue (data not shown) during the rest of the collections of apical shoots (June to April). Hence this short duration of availability of shoot buds immediately after the bud break limits and governed the entire process of somatic embryogeneis in *Pinus wallichiana*. During May (summer in India), the weather conditions (temperature 35±3°C) might be favorable for the significant vegetative growth of shoot buds indicating higher meristematic activity as compared against rest of the collections. Most of the buds were dormant during winter season (November-January in India) and failed to produce embryogenic tissue on the initiation medium even after the cold-pretreatment. After the winter season, most of the apical shoots undergone bud break and resumed vegetative growth during summer season (February-May in India). Shoot buds with higher meristematic activity and cold-pretreatment of thin sections has played an important role in the initiation of embryogenic tissue in all the three genotypes of *P. wallichiana*. These results are in agreement with previous investigations on somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus kesisya* (Malabadi *et al*., 2004a), *Pinus patula*

Table 1: Effect of various concentrations of smoke-saturated-water on somatic embryogeneis using vegetative shoot apices of *Pinus wallichiana* harvested during the month of May

| Genotypes | Smoke-water concentration in DCR basal medium (%) | Embryogenic cultures (%) | Total No. of somatic embryos recovered ($g^{-1}$FW embryogenic tissue) | Total No. of somatic embryos germinated ($g^{-1}$FW embryogenic tissue) | Total No. of somatic seedlings recovered ($g^{-1}$ FW embryogenic tissue) |
|---|---|---|---|---|---|
| Control[a] | 0 | 3.0±0.2c | 18.0±1.3b | 7.0±0.3c | 2.0±0.1c |
| PW10 | 10 | 13.0±1.2b | 37.0±1.5a | 35.0±2.4a | 30.0±2.7a |
| Control[b] | 0 | 4.0±0.6c | 14.0±1.2b | 6.0±0.3c | 3.0±0.1c |
| PW39 | 10 | 21.0±1.4a | 34.0±3.7a | 31.0±2.5a | 28.0±2.0a |
| Control[c] | 0 | 6.0±0.3c | 11.0±0.5b | 3.0±0.1c | 1.0±0.0c |
| PW120 | 10 | 27.0±2.1a | 39.0±2.4a | 33.0±2.6a | 27.0±1.8a |
| Control[a] | 0 | 3.0±0.2c | 18.0±1.3b | 7.0±0.3c | 2.0±0.1c |
| PW10 | 15 | 0 | 0 | 0 | 0 |
| Control[b] | 0 | 4.0±0.6c | 14.0±1.2b | 6.0±0.3c | 3.0±0.1c |
| PW39 | 15 | 0 | 0 | 0 | 0 |
| Controlc | 0 | 6.3±0.3c | 11.0±0.5b | 3.0±0.1c | 1.0±0.0c |
| PW120 | 15 | 0 | 0 | 0 | 0 |
| Control[a] | 0 | 3.0±0.2c | 18.0±1.3b | 7.0±0.3c | 2.0±0.1c |
| PW10 | 20 | 0 | 0 | 0 | 0 |
| Control[b] | 0 | 4.0±0.6c | 14.0±1.2b | 6.0±0.3c | 3.0±0.1c |
| PW39 | 20 | 0 | 0 | 0 | 0 |
| Control[c] | 0 | 6.0±0.3c | 11.0±0.5b | 3.0±0.1c | 1.0±0.0c |
| PW120 | 20 | 0 | 0 | 0 | 0 |

Control[a] = DCR basal medium without smoke-saturated water for genotype PW10, Control[b] = DCR basal medium without smoke-saturated water for genotype PW39, Control[c] = DCR basal medium without smoke-saturated water for genotype PW120, Data scored after 8 weeks and represent the mean±SE of at least four different independent experiments. In each column, values with different letters are significantly different ($p \leq 0.05$)

(Malabadi *et al*., 2003; Malabadi and Van Staden, 2003, 2004, 2005a-d, 2006), *Pinus roxburghii* (Malabadi and Nataraja, 2006), *Larix deciduas* Mill (Bonga, 1996, 2004) and *Larix* x *eurolepsis* (Bonga and Pond, 1991; Bonga and Von Aderkas, 1993).

The apical part of the shoot buds has yielded 2-3 thin sections and has resulted in the formation of embryogenic tissue on the initiation medium in all the three genotypes of *P. wallichiana*, whereas lower part of shoot buds produced non-embryogenic tissue. In all the genotypes, cold-pretreatment of apical shoot sections at 2°C incubated for 3 days on 0.3% activated charcoal produced white mucilaginous embryogenic tissue on initiation medium (Fig. 1B). Microscopic observations of embryogenic tissue revealed, elongated cells, some undergoing cleavage polyembryony. Concentrations of activated charcoal both lower than and higher than 0.3% decreased embryogenesis (data not shown). This study presents only optimum results. This clearly indicates lower concentrations of activated charcoal have a positive role on the initiation of embryogenic tissue. Charcoal provides a degree of darkness during *in vitro* culture. It is widely accepted that some of the beneficial effects of activated charcoal can be attributed to the removal of inhibitory substances from the media, produced either on autoclaving the media or released by the tissue itself. The percentage of embryogenesis was not similar in 3 genotypes. In case of control study, the highest percentage of embryogenic cultures (6%) was recorded in genotype PW120 (Table 1). The lowest percentage of embryogenic cultures (3%) was recorded in genotype PW10 (Table 1). Cold pretreatment has been used as an environmental stimulus to promote somatic embryogenesis in many plant species (Krul, 1993; Tamaszewska *et al*., 1994; Janeiro *et al*., 1995; Bonga, 1996, 2004; Malabadi and Van Staden, 2003, 2005a-d, 2006; Malabadi *et al*., 2004a; Malabadi and Nataraja, 2006). However, the role of cold-pretreatment in regulating the enhancement of somatic embryogeneis is not well understood (Malabadi *et al*., 2004a,b). Longer period of cold-pretreatments with higher percentage of activated charcoal in the media decreased the capacity for the formation of somatic embryos (Krul, 1993; Malabadi and Van Staden, 2003, 2005a-d, 2006; Malabadi *et al*., 2004a; Malabadi and Nataraja, 2006).

On the other hand the addition of SSW at a concentration of 10% in the medium (pre-culture, initiation, maintenance, maturation and germination) has increased the percentage of somatic embryogenesis in all the three genotypes of *P. wallichiana* as compared against control (Table 1) (Fig. 1C -F). Lower concentrations of SSW do not have any effect on embryogenesis (data not shown). Higher concentrations (15 and 20%) of SSW inhibited somatic embryogenesis in all the three genotypes of *P. wallichiana* (Table 1). Therefore, addition of 10% of SSW in the DCR basal medium was found to be the optimum concentration for the entire process of embryogenesis in *P. wallichiana* (Table 1). At high concentrations, smoke extracts are known to inhibit seed germination and more dilute solutions improved the germination in dormant seeds of *Syncarpha vestita* (L.) B. Nord (Brown *et al*., 2003). The highest percentage of embryogenic cultures (27%) was recorded in the genotype PW120. Lowest percentage of embryogenic cultures (13%) was recorded in the genotype PW10 as compared against control (Table 1). The presence of SSW in maturation medium increased the rate of embryo development as evidenced by the occurrence of more number of well matured somatic embryos (PW-37; PW-34; PW120-39) recovered per gram fresh-weight of embryogenic tissue as compared against control (Table 1). In the best treatment of SSW, 39 somatic embryos of PW120 genotype were at cotyledonary stage compared to 11 in untreated controls (Fig. 1E) (Table 1). The germination of somatic embryos in all the three genotypes of *P. wallichiana* was promoted by the presence of SSW in the germination medium as compared against control (Table 1) (Fig. 1F). Maximum number of somatic embryos germinated successfully and resulted in the recovery of maximum seedlings compared against control (Table 1). SSW treated somatic seedlings showed higher percentage of seedling survival. The physiological mechanism resulting in improved vigour is unknown. However, smoke water may protect the seed and seedlings against microbial attack which can result in higher seedling survival (Light and Van Staden, 2004). The recent identification of the germination cue from smoke will now

allow for research into the physiological action of smoke on seed germination. SSW does not have any significant effect on the germination period of somatic embryos in all the three genotypes of *P. wallichiana*. Both SSW treated and untreated (control) somatic embryos have taken same days (3 weeks) for the germination. Therefore, SSW has affected the total number of somatic embryo germination but not the germination time (data not shown).

In case of geranium, *Pelargonium hortorum* Bailey cv Elite, SSW treatment (10%) of the explant prior to induction, or together with the inductive signal (TDZ) produced the highest number of somatic embryos. These observations suggest that the active ingredient (s) in SSW play a regulatory role in plant development. The number of somatic embryos doubled following the addition of SSW at either explant or induction stage compared to the untreated control (Senaratna *et al*., 1999). This study suggests that SSW may affect the process of somatic embryogenesis in a manner analogous to a plant growth regulator. Somatic embryogenesis is initiated in response to a chemical signal (s) which generally are growth regulators, which in turn alters the endogenous auxin and cytokinin concentration, with the auxin: cytokinin ratio suggested critical factor in the induction of embryogenic competence. The inductive signal for the initiation of somatic embryogenesis of *P. wallichiana* used in this present study were BAP, NAA and 2, 4-D. Smoke-saturated-water (without BAP, NAA and 2, 4-D) did not induce any form of cell proliferation; however SSW appeared to act synergistically with the inductive signal. Collectively taken, these observations suggest that SSW acts like a growth regulator than a nutritional additive. It has been suggested that smoke may have an action similar to cytokinins in breaking celery seed dormancy (Thomas and Van Staden, 1995). In present study the presence of SSW in the medium has a significant effect on the different developmental stages of somatic embryogenesis of *P. wallichiana* (Fig. 1B-F). However, at this stage it is very difficult to conclude the role of SSW on somatic embryogenesis. Whether the stimulatory role of SSW in somatic embryogenesis is similar to cytokinin-like action or smoke-water interacts with any other growth regulators involved in somatic embryogenesis warrants further investigation (Senaratna *et al*., 1999). This study shows that application of smoke technology can be adopted to produce more number of healthier and vigorous somatic seedlings for the commercial forestry.

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