

## Short communication. Factor influencing *in vitro* seed germination and correlation of antioxidant activity with tissue development in *Cedrus deodara*

N. Ahmad<sup>1\*</sup>, H. Fazal<sup>2</sup>, B. Haider Abbasi<sup>1</sup> and M. Ali<sup>1</sup>

<sup>1</sup> Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, 45320 Islamabad, Pakistan

<sup>2</sup> Pakistan Council of Scientific and Industrial Research (PCSIR), Laboratories Complex, 2500 Peshawar, Pakistan

### Abstract

**Aim of study:** The main objective of this study is to introduce a reliable system for *in vitro* seed germination. *Cedrus deodara* stands are gradually decreasing in different regions of the world due to overexploitation, natural disasters and lower seed viability.

**Area of study:** Swat is situated at the northwestern corner of Pakistan; with total area of 5,337 square km. Total cultivated land is 95281 hectares in 2007, while the area under forest cover is 135,427 hectares.

**Material and methods:** We used MS-medium with or without PGRs for overall *in vitro* seed germination. To enhance germination frequency, we applied different photoperiods (16hrs-Dark/8hrs-Light and 16hrs-Light/8hrs-Dark) and sterilization reagents (mercuric chloride and ethanol). Synthetic free radical of DPPH (1, 1-diphenyl-2-picrylhydrazyl) was used for the determination of antioxidant activity

**Main results:** Maximum shoot length (3.6 cm) and root length (2.6 cm) were recorded on MS-medium augmented with BA (1.0 mg L<sup>-1</sup>) and GA<sub>3</sub> (0.5 mg L<sup>-1</sup>) under light incubation (16 hrs-Light/8hrs-Dark) after 2-3 weeks of inoculation. Without PGRs, maximum root length (4.0 cm, with shoots of 3.2 cm) was observed in dark incubation (16 hrs-Dark/8 hrs-Light), whereas light incubation produced maximum shoot length (3.5 cm) and minimum root length (1.5 cm). Lower concentration of HgCl<sub>2</sub> (0.1%) showed a lower inhibitory effect on shoot and root length (2.4 cm and 2.5 cm) as compared to higher concentrations. The antioxidant potential was also investigated in different *Cedrus* organs and tissues.

**Research highlights:** The results suggested that this simple protocol is useful for *Cedrus deodara* conservation and plantlets production for commercial purposes.

**Key words:** *Cedrus deodara*; seed germination; callus; 6-Benzyladenine; antioxidant.

### Introduction

*Cedrus deodara* is a Pinaceae species distributed in the western Himalayas and some Mediterranean regions (Chaudhary *et al.*, 2011). Natural disasters and overexploitation have depleted genetic resources of the species in the last decades. Despite other uses, this species produced a variety of active compounds of himachalol, atlantone, trans-atlantone, matairesinol,

nortrachelogenin, dibenzylbutyrolactollignan and ecteoside which showed proven antioxidant and anticancer activity (Chaudhary *et al.*, 2011; Tiwari *et al.*, 2001; Saxena *et al.*, 2010). These compounds stop the chain reaction of free radicals that damage cellular components of DNA, proteins and lipids and produced serious diseases including lethal mutation (Ahmad *et al.*, 2011). Tiwari *et al.* (2001) extracted different compounds from wood which showed higher DPPH

\* Corresponding author: [nisarbiotech@gmail.com](mailto:nisarbiotech@gmail.com)

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Abbreviations: MS: Murashige and Skoog medium; MS0: Murashige and Skoog medium without PGRs; PGRs: Plant Growth Regulators; BA: 6-Benzyladenine; GA<sub>3</sub>: gibberellic acid; HgCl<sub>2</sub>: Mercuric chloride; DPPH: 1-diphenyl-2-picrylhydrazyl; mg L<sup>-1</sup>: milligram per litre; g L<sup>-1</sup>: Gram per litre; nm: Nanometer; CRBD: Complete Randomized Block Design; DMRT: Duncan's multiple range tests.

activity. Saxena *et al.* (2010) isolate a novel antioxidant (lignan) from this species which showed anticancer properties against human leukemia. Recently Ahmad *et al.* (2013b) reported that natural antioxidants can be used for regeneration of damaged hepatic tissues.

Seeds of *C. deodara* lose viability when stored under normal conditions due to its resin (Rudolf, 1974; Allen, 1995). The viability can be raised to 6 years if the moisture content is reduced to 10% at  $-5$  to  $-1^{\circ}\text{C}$  (Rudolf, 1974; Erkuloglu and Kayin, 1995).

Germination of seeds under controlled conditions (*in vitro*) can reduce the time required for production of plantlets, passing harsh conditions and pathogen attacks (Nikolic *et al.*, 2006). Therefore the main objective of this study is to investigate the effect of light-dark cycle on seed germination under *in vitro* conditions. Callus induction and antioxidant activity were also determined. To the best of our knowledge, germination of seeds on MS-medium and the antioxidant activity in different *in vitro* grown tissues is reported for the first time in this specie. We have established a reliable protocol for seed germination which can be also used for other related species.

## Materials and methods

### Seed collection and germination under light-dark cycles

Seeds of *C. deodara* were taken from Forest Department, Behrain Swat, Pakistan. For surface sterilization, ethanol (70%; v/v) and  $\text{HgCl}_2$  (0.2%; w/v) were used following the methods of Ahmad *et al.* (2013a; 2013b). These seeds were then inoculated on MS medium (Murashige and Skoog, 1962) augmented with sucrose ( $30\text{ g L}^{-1}$ ), solidified with agar ( $8\text{ g L}^{-1}$ ) (Sigma) at pH 5.6-5.8. For dark (16h rs-Dark/8 hrs-Light) and light photoperiod (16hrs-Light/8hrs-Dark) the MS-medium was used without PGRs. Another experiment was designed in which BA and  $\text{GA}_3$  were added to the MS-medium with light photoperiod to enhance germination. All media were sterilized at  $121^{\circ}\text{C}$  for 20 min. The cultures were maintained for 6-7 weeks  $25 \pm 2^{\circ}\text{C}$  in growth room.

### Sterilization reagents

Different concentrations of sterilization reagents were studied to check the survival rate of the seeds.

Seeds were soaked for two hours in mercuric chloride (0.1-0.5%) and ethanol (60-100%). Under *in vitro* conditions the seeds were removed from treatment reagents. Without washing they were dried using filter paper. The seeds were inoculated on MS-medium without adding any PGRs. The survival rate and viability of seeds were checked against dose dependent concentration. The flasks were kept in growth room at  $25 \pm 2^{\circ}\text{C}$ , under normal photoperiod (16hrs-Light/8hrs-Dark), data was collected after 5-6 weeks.

### Antioxidant activity

Antioxidant activity was determined according to the method of Ahmad *et al.* (2013b). 10.0 mg of dried plant tissues was soaked in 4 ml methanol, mixed with a methanolic solution of DPPH (1 mM, 0.5 mL). This mixture was incubated for 30 min and the absorbance was recorded at 517 nm. The percentage DPPH activity for each tissue was determined by using the equation;

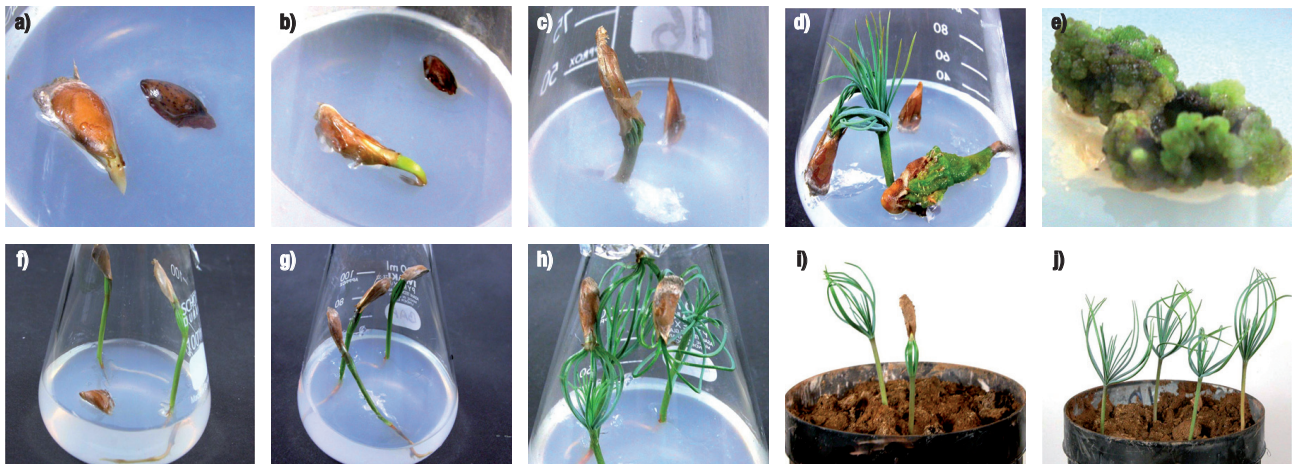
$$\% \text{ scavenging DPPH}^{\circ} \text{ free radical} = 100 \times (1 - A_E/A_D)$$

In the equation  $A_E$  is used for the solution (Sample + DPPH) absorbance, and  $A_D$  is used for the DPPH $^{\circ}$  absorbance at 574 nm.

**Table 1.** Effect of different factors on seed germination of *Cedrus deodara*

Factors	Germination (%)	Days to germination
BA ( $0.5\text{ mg L}^{-1}$ )	$33.40 \pm 3.40^{\text{g}}$	30 days
BA ( $1.0\text{ mg L}^{-1}$ )	$35.17 \pm 4.17^{\text{g}}$	30 days
BA ( $1.5\text{ mg L}^{-1}$ )	$40.51 \pm 4.51^{\text{fg}}$	30 days
BA ( $2.0\text{ mg L}^{-1}$ )	$43.92 \pm 3.92^{\text{f}}$	30 days
BA ( $0.5$ ) + $\text{GA}_3$ ( $0.5\text{ mg L}^{-1}$ )	$63.66 \pm 5.66^{\text{de}}$	28 days
BA ( $1.0$ ) + $\text{GA}_3$ ( $0.5\text{ mg L}^{-1}$ )	$78.45 \pm 1.55^{\text{a}}$	21 days
BA ( $1.5$ ) + $\text{GA}_3$ ( $0.5\text{ mg L}^{-1}$ )	$70.61 \pm 2.61^{\text{c}}$	25 days
BA ( $2.0$ ) + $\text{GA}_3$ ( $0.5\text{ mg L}^{-1}$ )	$70.34 \pm 5.34^{\text{c}}$	25 days
16 hrs-Light/8 hrs-Dark	$75.90 \pm 5.90^{\text{ab}}$	24-28 days
16 hrs-Dark/8 hrs-Light	$72.12 \pm 5.12^{\text{bc}}$	27-31 days
Mercuric chloride (0.1%)	$33.32 \pm 3.33^{\text{g}}$	35 days
Mercuric chloride (0.2%)	$30.19 \pm 5.19^{\text{h}}$	35 days
Mercuric chloride (0.3%)	$23.78 \pm 3.78^{\text{ij}}$	38 days
Mercuric chloride (0.4%)	$20.93 \pm 2.93^{\text{j}}$	38 days
Mercuric chloride (0.5%)	$20.34 \pm 2.34^{\text{j}}$	38 days
Control (MS0)	$66.66 \pm 4.66^{\text{c}}$	45 days

\* Data (mean  $\pm$  SD + LSD) was obtained from three independent experiments. Mean values are significantly different when  $p < 0.01$ .



**Figure 1.** *In vitro* seeds germination of *C. deodara*. (a-c) Initiation of seeds germination. (d-e) Callus induction. (f-h) Shooting and rooting. (i-j) Acclimatization.

### Statistical analysis

The statistical analysis was carried out according to Complete Randomized Block Design (CRBD). Eight cultured flasks were used per treatment and the data were documented from triplicates. Duncan's multiple range test (DMRT) was used to compare treatment mean.

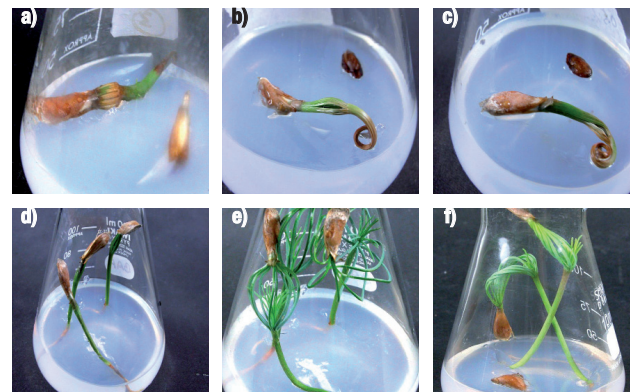
### Results and discussion

In this study the highest germination (78.45%) was observed when MS medium was augmented with BA ( $1.0 \text{ mg L}^{-1}$ ) and GA<sub>3</sub> ( $0.5 \text{ mg L}^{-1}$ ) followed by light incubation (75.90%) as shown in Table 1. However, different concentrations of BA and HgCl<sub>2</sub> showed lower germination than control. Significantly higher shoot/root length ratios were recorded on MS medium supplemented with 1.0-2.0 mg L<sup>-1</sup> BA along with  $0.5 \text{ mg L}^{-1}$  GA<sub>3</sub> (Figs. 1, 3). These results are consistent with those of Nikolic *et al.* (2006) and Bhattacharya and Khuspe (2001), who reported that most of the cytokinins including BA and TDZ influenced seed germination in *Lotus corniculatus* and *Carica papaya*. However, seeds were also placed on control MS<sub>0</sub> (MS-medium without PGRs) which showed germination after 38-45 days.

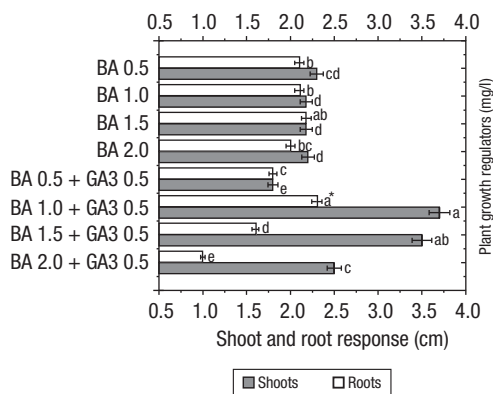
We concluded from this study that light and dark cycles (photoperiods) significantly affect the seed germination rate of *C. deodara* on MS-medium without any PGRs (Table 1). Yellowish green shoots (3.2cm) were observed under dark incubation, probably due to reduced chlorophyll contents (Fig. 2-F). However, the

maximum root length (4.0 cm) was recorded under dark incubation (Fig. 4). As compared to dark incubation, highest shoot length of 3.5cm was recorded when seeds were incubated in light for four weeks (Fig. 4). The light effect enhances shooting from seeds. This effect increases when the medium was supplemented with BA and GA<sub>3</sub> (Fig. 2-e). Similarly, Bhattacharya and Khuspe (2001) reported that *in vitro* conditions along with light-dark incubation is very effective for seed germination in different cultivars of papaya.

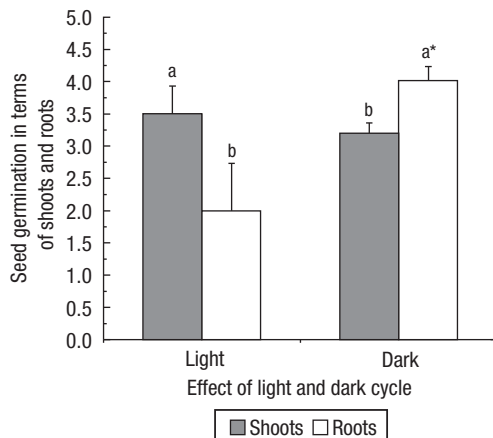
Lower concentrations (0.1%) of HgCl<sub>2</sub> solution produced shoot length of 2.4 cm (Fig. 5), while higher concentrations (0.5%) were found toxic for germination (Fig. 2a). Similarly best rooting (2.2 cm) was observed with 0.1% HgCl<sub>2</sub>, but with higher concentrations the roots started curling and browning (Fig. 2b,c).



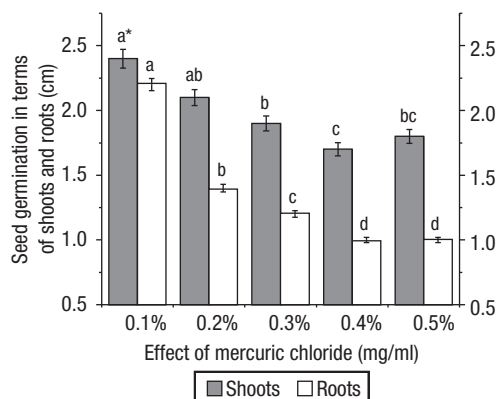
**Figure 2.** (a) Effect of 0.5% HgCl<sub>2</sub> on seeds germination. (bc) Effect of 0.3% and 0.4% HgCl<sub>2</sub> on seeds germination. (de) Effect of light on shoot and root response. (f) Shoot and root response in dark.



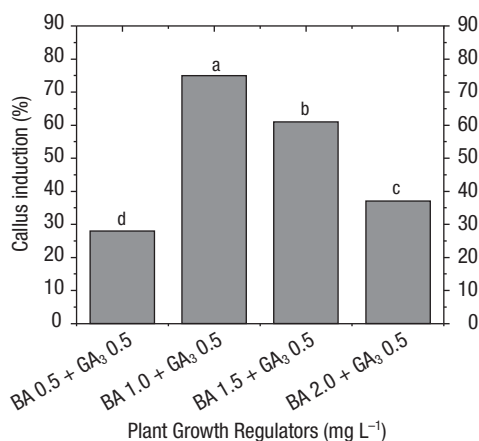
**Figure 3.** Effect of various concentrations of BA and GA<sub>3</sub> on seed germination. Values (mean + SD) were taken from three independent experiments. Mean values with common letters (\*) are significantly different at  $p < 0.05$ .



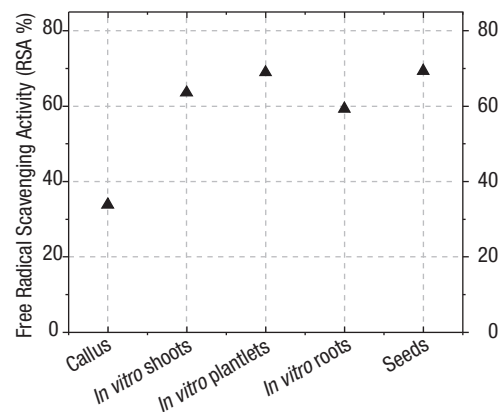
**Figure 4.** Effects of light and dark incubation cycle on shoot and root response. Mean values in each column with common letters (\*) are significantly different at  $p < 0.05$ .



**Figure 5.** Effects of mercuric chloride on germination. Mean values in each column with common letters are significantly different at  $p < 0.05$ .



**Figure 6.** Effects of various PGRs on percent callus induction in *C. deodara*. Data was collected after 4 weeks of culture. Values are means of 3 replicates with SD Bars with common letters are significantly different at  $p < 0.05$ .



**Figure 7.** Antioxidant activity in different tissues and organs of *C. deodara*.

In the present investigation different concentration (60-90%) of ethanol was also used. However as compared to mercuric chloride, ethanol penetrates easily across seeds membrane, causing seeds death. It was observed that no seeds were germinated when ethanol was applied. Similarly, the findings of Maina *et al.* (2010) are in agreement with our data that Mercuric chloride is less harmful than other sterilants.

In this study callus was obtained (75%) accidentally when germinated seedling was placed for longer period on MS-medium containing a combination of BA and GA<sub>3</sub> (Fig. 6 and 1-de). Ahmad *et al.* (2013a) also reported that BA and GA<sub>3</sub> significantly influenced callus induction in *Piper nigrum*. A higher antioxidant (DPPH) potential was found in the seeds and *in vitro*

plantlets (<68%) than *in vitro* shoots, roots and callus cultures of *C. deodara* (Fig. 7). The current data are in agreement with the results of Khan *et al.* (2013), who reported similar effect of BA and GA<sub>3</sub> on antioxidative enzyme activities. The antioxidant activities in *Cedrus* tissues under different photoperiods are not available in the literature.

The data presented in this report show that combination of BA and GA<sub>3</sub>, and incubation of seeds in light and dark photoperiods significantly enhanced germination parameters in *C. deodara* (Table 1). Furthermore, the antioxidant activity showed that *in vitro* plantlets and seeds of this species can be used for the treatment of serious diseases.

## Acknowledgements

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