Quantification of taxol by high-performance thin layer chromatography in *Taxus wallichiana* callus cultivated *in vitro*

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Abstract

In the present study, the induction of callus, callus biomass growth and the yield of taxol were investigated in *Taxus wallichiana*. This is the first report of a quantification study of taxol in *in vitro* grown tissues of *Taxus* obtained from Jammu and Kashmir provinces, India. For callus induction, three different explant types (leaf, cone and stem) were cultured in media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). The stem was most responsive to callusing, and the maximum callus induction frequency of 71.5% was noted on medium amended with 2.0 mg/l 2,4-D. The medium amended with 2,4-D and ascorbic acid (AA) showed better callus growth and the maximum biomass (0.082 g, fresh weight). The yield of taxol was quantified in the callus grown on a medium supplemented with different plant growth regulators (PGRs) by using high-performance thin layer chromatography (HPTLC). The taxol yield was maximum (1.053 µg/g dry weight) in 2,4-D-stimulated callus, followed by callus treated with NAA in which 0.896 µg/g dry weight taxol was detected. The maximum taxol yield (56.6%) was obtained in the callus grown on the medium amended with 2,4-D, and a 33.3% increased yield was noted on the NAA-supplemented medium. As a stress marker, the activities of the antioxidant enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) and the level of proline were measured in an auxin (2,4-D and NAA)-supplemented medium. The callus grown on the 2,4-D-supplemented medium had high levels of SOD (3.91 $min^{-1} \cdot mg^{-1}$ protein), APX (1.61 $min^{-1} \cdot mg^{-1}$ protein) and proline (6.57 mg/g), thus suggesting a higher stress level; the callus grown on the NAA-supplemented medium had slightly lower levels of SOD and APX enzyme activities and proline content (3.01 $min^{-1} \cdot mg^{-1}$ protein, 1.04 $min^{-1} \cdot mg^{-1}$ protein and 5.90 mg/g, respectively). BAP had little influence on stress parameters. The present study thus indicates a good taxol yield in the callus cultured in 2,4-D, which functioned as a signalling element and a stressor. The taxol yield in response to PGRs was analysed in *in vitro* cultivated tissues.

Key words: antioxidant enzymes, callus induction, stress, taxol, *Taxus wallichiana*, 2,4-dichlorophenoxyacetic acid

Introduction

Taxus is an important genus of the family *Taxaceae.* It is a slow-growing tree. There are nine different species of *Taxus,* and some of the more important ones are *Taxus sumatrana, T. globosa, T. baccata, T. brevifolia* and *T. canadensis.* The latter two, i.e. *T. brevifolia* and *T. canadensis*, are important sources of paclitaxel or taxol–the chemotherapeutic drug used against a variety of cancers (Spjut, 2007; Howat et al., 2014; Zhu and Chen, 2019). Other species of *Taxus* such as *T. walli-* *chiana* also produce an analogous compound named docetaxel with similar anticancer activities (Vobořilováa et al., 2011). *T. wallichiana*, called Himalayan Yew, is native to Himalaya; the plant is also found in China, Nepal, Bhutan, Myanmar, Vietnam, the Philippines and Sumatra. Besides its ornamental value, *T. wallichiana* has been used in Ayurveda, Tibetan and other traditional medicines as well as in making tea and as a fuel wood in tribal, local communities (Itokawa and Lee, 2002; Juyal et al., 2014).

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Taxus has been indiscriminately used across its source regions for its immensely valuable leaves and bark. This pilferage is more alarming in India and other neighbouring countries. A whopping 90% loss has been reported in recent times, and therefore, *Taxus* was declared as an endangered species by IUCN (Thomas and Farjon, 2011; Coughlan et al., 2020). Efforts have been made to conserve and propagate *Taxus* sp. to identify more compounds with anticancer properties and to enrich the level of alkaloids for commercial application (Abbasin et al., 2010; Lichota and Gwozdzinski, 2018). Together with the use of natural sources, the production of synthetic taxol has been attempted with fair success, but the commercial production of taxol has not yet been achieved as the synthetic pathway is very long and complicated, and requires several intermediates and enzymes, thus increasing the cost of production (Badi et al., 2015). Semi-synthesis of taxol is also possible, but the success partly depends on the availability of the natural molecule Bacatin III which is limited (Commercon et al., 1995). As an alternative, plant cells, tissues, organs and suspensions are widely used for enriching alkaloids, apart from their use in mass multiplication of plants for raw materials (Abbassin et al., 2010). An additional advantage of this approach is that plant biotechnology can significantly improve the yield of compounds when compared with that of intact plants. In recent times, various strategies have been used to improve alkaloid production in *in vitro* culture; some of these strategies include optimization of the medium composition and plant growth regulators (PGRs), addition of precursors, overexpression of key enzymes that regulate product synthesis, and use of biotic and abiotic compounds that induce stress (Murthy et al., 2014; Maqsood and Mujib, 2017). Factors inducing cellular stress include ultraviolet (UV) rays, heavy metals, adverse climate, salinity caused by various compounds such as NaCl and $CaCl_2$, and osmotic stress caused by various elements including PGRs (Elmaghrabi et al., 2013; Samar et al., 2015; Dipti et al., 2016). The influence of PGRs on callus induction, biomass accumulation and alkaloid synthesis has been reported in various models and medicinal plants (Teixeira and Dobranszki, 2016). In *Daucus carota*, PGRs such as 2,4-dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA) and naphthaleneacetic acid (NAA) improved callus growth and anthocyanin accumulation in the culture, while the cytokinin kinetin (Kin) alone or in

combination with IAA and methyl jasmonate was noted to be very effective in promoting callus biomass and alkaloid accumulation (Narayan et al., 2005). Raj et al. (2015) conducted a comprehensive study on the role of PGRs on alkaloid accumulation in *in vitro* culture of *Securinega suffruticosa* by using several different auxins and cytokinins, and the combination of IAA (0.5 mg/l) and Kin (5.0 mg/l) was observed to be effective for the synthesis of securinine and allosecurinine. PGRs regulated the accumulation of phytocompounds such as isoflavone, carotenoids and chlorophyll, as noted in a recent study of *Genista tinctoria* and *Lamprocapnos spectabilis* plant cell culture (Luczkiewicz et. al., 2014; Kulus, 2020) although the synthesis of alkaloids is often species- and dose-dependent (Ramawat and Mathur, 2007). When added to the medium, these PGRs induce cellular stress and are used as signalling molecules to trigger a cascade of gene expression in biosynthetic pathways of alkaloid synthesis (Binder et al., 2009; Zahid and Mujib, 2012; Mujib et al., 2016). The biotic/abiotic stress induced in cultured tissues is assayed by investigating the responses of antioxidant enzymes in cultured cells (Cai et al., 2012; Nadia et al., 2017). Stress marker enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) are studied to estimate stress levels in cultivated plant cells (Samar et al., 2015).

In the present study, a protocol for rapid initiation and multiplication of callus in *T. wallichiana* was established. The yield of taxol in the callus was quantified by High-Performance Thin Layer Chromatography (HPTLC) and the correlation of the stress level with the increased yield of taxol was established by assessing stress markers. The role of auxins in enriching the taxol yield was also evaluated.

Materials and methods

Plant material

Juvenile newly grown twigs of *Taxus wallichiana* Zucc. were collected from Jammu and Kashmir provinces, India, in April-May 2016. Three different explants, i.e. leaf, stem and miniature cone, were tested for their ability to induce callus. The explants were first surface-disinfected with 0.5% (w/v) $HgCl_2$ for 5 min and then washed 3–4 times with sterilized distilled water. The explants, i.e. leaf, stem and cone pieces (8–12 mm long), were individually placed in a test tube containing 15 ml

of MS medium (Murashige and Skoog, 1962) containing macro-salts, micro-salts, iron, vitamins, myo-inositol (100 mg/l), glycine (2.0 mg/l) and 30 g/l sucrose. The medium was also supplemented with various PGRs such as 2,4-D, NAA and 6-benzylaminopurine (BAP) at the concentrations of 0.5, 1.0, 2.0 and 4.0 mg/l each. The pH of the medium was maintained at 5.7, and the medium was sterilized by autoclaving at $121\,^{\circ}$C for 15 min. The different reagents were prepared with water provided by the Milli-Q system (Billerica, MA, USA). The cultures were kept at $25\pm2\,^{\circ}$C for a 16-h illuminated photoperiod of cool white fluorescent tubes (100 μmol$\cdot$m$^{-2}\cdot$s^{-1} Photon Flux Density, [PFD]).

Callus biomass growth

Thirty milligrams of induced callus was weighed and cultured on MS medium as the initial inoculum. Two different auxins, namely 2,4-D (2.0 mg/l) and NAA (2.0 mg/l), were used separately with ascorbic acid [AA] (0.5 mg/l) for callus biomass growth and for preventing browning. Fresh weight of the increased callus was taken after 4th, 8th and 12th weeks of incubation. The cultures were kept in the same culture regime unless mentioned otherwise.

Taxol quantification

Method of extraction

Taxol was extracted from callus tissues following the protocol of Junaid et al. (2010), and the yield of taxol was quantified in callus tissues of different ages (4 to 16 weeks old). The calli were harvested separately from media containing 2,4-D, NAA and BAP (2.0 mg/l) after adequate growth and proliferation of these tissues. For comparison, callus was harvested from a PGR-free medium and designated as control callus. *In vivo* grown leaves and stems were also included as a study material, and the yield was compared with that of the standard taxol obtained from Sigma-Aldrich (St. Louis, MO, USA). Approximately one gram of callus was used and incubated for 5 h in 30 ml of methanol; the supernatant was boiled at $60\,^{\circ}$C, and the final volume was made to 1.0 ml.

Standard taxol stock solution and calibration curve

One milligram of taxol was dissolved in 1.0 ml methanol to make 1.0 mg/ml stock solution. Different concentrations, i.e. 200, 400, 600, 800 and 1000 μg/ml, of taxol were prepared from stock preparations and were

run in HPTLC to construct the standard curve. The curve generated between the peak area on the y-axis and standard taxol concentrations on the x-axis showed a good linear relationship with $r=0.998$.

HPTLC instrumentation and taxol quantification in the callus

An aluminium-coated TLC system (60 F 254, Merck) of 20×10 cm size (with silica gel) was used. The mobile phase consisted of toluene, carbinol, acetone and ammonia in the ratio of $40:20:80:2$, respectively. The callus extracts were applied through a 100 μl syringe using Linomat 3 (CAMAG) applicator. The air-dried silica plates were placed in a chamber (twin-trough chamber [CAMAG], 20×10 cm) containing the mixture of the mobile phase. The solvent solution moved upward and was allowed to climb up to a distance of approximately 85 mm. Following this, the TLC plates were removed and air-dried for another 10–20 min. The silica plates were kept in CAMAG Reprostar under UV light. The taxol-containing phase was recorded by CAMAG Scanner 3 by scanning at 295 nm. Taxol was quantified by comparing the peaks of taxol with those of the standard sample. The yield was measured in μg/g dry weight basis.

SOD assay

The SOD activity was estimated according to the method of Dhindsa et al. (1981). Callus tissues (0.1 g) were homogenised in 2.0 ml of extraction solution containing 0.5 M sodium phosphate buffer, pH 7.3; 3.0 mM EDTA; 1.0% (w/v) polyvinylpyrollidone (PVP); and 1.0% (v/v) Triton X-100; the entire mixture was centrifuged at $4\,^{\circ}$C, 10000 rpm for 10 min. The SOD activity was estimated by observing inhibition of the photochemical reduction. The SOD assay mixture comprised 1.5 ml reaction buffer, 0.2 ml methionine, 0.1 ml enzyme extract, 1.0 M NaCO$_3$, 2.25 mM Nitro Blue Tetrazolium (NBT) solution, 3.0 mM EDTA, riboflavin and 1.0 ml H$_2$O. The mixture was poured in test tubes and kept at $25\,^{\circ}$C under light for 10 min. A 50% colour loss measured spectrophotometrically was considered to be 1.0 unit, and the enzyme concentration was expressed as Enzyme Unit (EU) mg^{-1} protein min^{-1}.

APX activity

To determine APX activity, the method of Nakano and Asada (1981) was followed. The assay mixture comprised 1.0 ml (0.1 M) sodium buffer (pH 7.2), 0.1 ml

EDTA and 0.1 ml enzyme extract. The ascorbate was incorporated, and the reaction was continued for 3 min at 25°C. The APX activity was estimated spectrophotometrically by a decrease in absorbance at 290 nm and using the absorption coefficient of 2.8 mM$^{-1}\cdot$cm^{-1}. The activity was measured as EU mg^{-1} protein min^{-1}, i.e. a single unit of enzyme is the minimum amount required to decompose 1.0 μmol of ascorbate per minute.

Proline level estimation in the extract

For proline quantification, the method of Bates et al. (1973) was followed. Callus tissues (0.2 g) were ground in 5.0 ml aqueous sulfosalicylic acid (3%) and filtered through a Whatman filter paper No. 1 (Camlab, UK). Approximately 1.0 ml acid ninhydrin and 1.0 ml glacial acetic acid were added to 1.0 ml tissue extract; the mixture was incubated at 100°C for 1 h. Finally, the reaction solution was kept on ice and was extracted with 2.0 ml toluene. The spectrophotometric assay was conducted for measuring the proline level in the extract and was expressed as mg/g unit.

Statistical analysis

The effect of PGRs on callus induction in various explants, callus biomass growth, differences in biochemical parameters such as enzyme activity, protein markers and taxol yield were monitored and analysed statistically. In each case, the experiments were performed in triplicates which were conducted at least twice. For callus induction, the data were scored after 4 weeks of culture. The data are expressed as mean values and were compared using the Duncan's Multiple Range Test (DMRT); differences were considered to be significant at $P \leq 0.05$.

Results

Callus induction and biomass growth

Three different explants of *T. wallichiana* were tested for their ability to produce callus in MS media supplemented with different PGRs. In almost all the tested PGR concentrations, callus was induced from plant parts at varying intensities (Table 1). The stem was found to be more responsive than leaf and cone explants. On MS with 2.0 mg/l 2,4-D, more than 71% of the cultured stems responded by producing callus (Fig. 1A, Fig. 1B). The stem also produced callus in the NAA-containing

medium, but was less vigorous, with the maximum callus induction percentage of 59%. The effect of BAP supplementation was observed to be less as only 2 to 12% of stems developed callus. Therefore, the experiment on the addition of BAP into the medium was discontinued. The callus started to appear within 2 weeks of culture on 2,4-D-added MS and was yellowish green, granular, compact and slow-growing. With time, the callus turned brown (Fig. 1C), and sub-culturing of the callus was then performed at regular intervals of 2–3 weeks to prevent browning. In other cultures, AA was added to the medium to make the callus healthy for a prolonged period of time by inhibiting necrosis/browning (Fig. 1D). The stem-induced calli were cultivated in an optimized 2,4-D (2.0 mg/l) and NAA (2.0 mg/l)-added medium with or without AA, and the callus biomass growth was monitored (Fig. 2). Callus growth is an indicator of cell division, which increases or decreases with time. In 2,4-D and AA-amended media, the callus growth was only slightly higher (0.082 g, fresh weight) than that in the medium devoid of AA (0.080 g, fresh weight). The callus biomass grew slowly with time, and after 12 weeks of incubation, the maximum fresh weight (0.082 g) was observed. A relatively poor callus growth was observed in NAA (2.0 mg/l) and AA-amended medium (0.070 g, fresh weight) when compared with that in the medium amended with 2,4-D (2.0 mg/l) and AA.

Taxol yield

The yield of taxol was measured in callus tissues at periodic intervals (4 weeks) (Fig. 3). The standard taxol in the mobile phase showed a clear sharp peak (Fig. 4). The results presented in Table 2 show that the taxol yield was higher in 2,4-D-containing medium (1.053 μg/g dry weight) than in the control (0.672 μg/g dry weight). The taxol yield was marginally high (0.896 μg/g dry weight) in medium containing NAA (2.0 mg/l). The level was very low in BAP-supplemented MS medium. The maximum increase in taxol yield (by 56.69%) was observed in 2,4-D-fortified medium, followed by that in NAA-supplemented medium (33.33% increase) over the control callus. The taxol yield showed a steady increase with the callus age and was maximum at the 12th week of culture (Fig. 2); however, the taxol yield declined with time as further passages were performed.

Table 1. Callus induction frequency [%] of *Taxus wallichiana* from various explants
in media supplemented with different PGRs [mg/l]

PGR [mg/l]		Leaf	Cone	Stem
2,4-D	0.5	21.52 ± 2.55 [c]	4.85 ± 0.82 [d]	35.15 ± 5.55 [d]
	1.0	31.42 ± 3.62 [b]	6.85 ± 1.11 [c]	56.15 ± 6.17 [b]
	2.0	36.50 ± 2.85 [a]	12.12 ± 0.96 [a]	71.58 ± 5.25 [a]
	4.0	12.81 ± 1.88 [d]	8.16 ± 1.06 [b]	40.20 ± 4.28 [c]
NAA	0.5	17.62 ± 2.88 [c]	4.21 ± 0.88 [b]	31.12 ± 4.44 [c]
	1.0	26.21 ± 2.22 [b]	4.16 ± 0.67 [b]	41.16 ± 4.48 [b]
	2.0	32.15 ± 3.86 [a]	6.18 ± 1.12 [a]	59.25 ± 4.65 [a]
	4.0	10.16 ± 1.18 [d]	6.10 ± 1.22 [a]	32.56 ± 3.8 [c]
BAP	0.5	0 [c]	0 [c]	0 [d]
	1.0	2.16 ± 0.85 [b]	2.12 ± 0.17 [b]	10.85 ± 2.11 [b]
	2.0	5.12 ± 1.17 [a]	3.20 ± 0.85 [a]	12.16 ± 2.35 [a]
	4.0	0 [c]	0 [c]	7.86 ± 1.60 [c]

The data were analysed after 4 weeks of cultivation; values are expressed as mean ± standard error of three replicates of two experiments; within the column, values followed by a different letter are significantly different at $P \leq 0.05$ according to the Duncan's Multiple Range Test
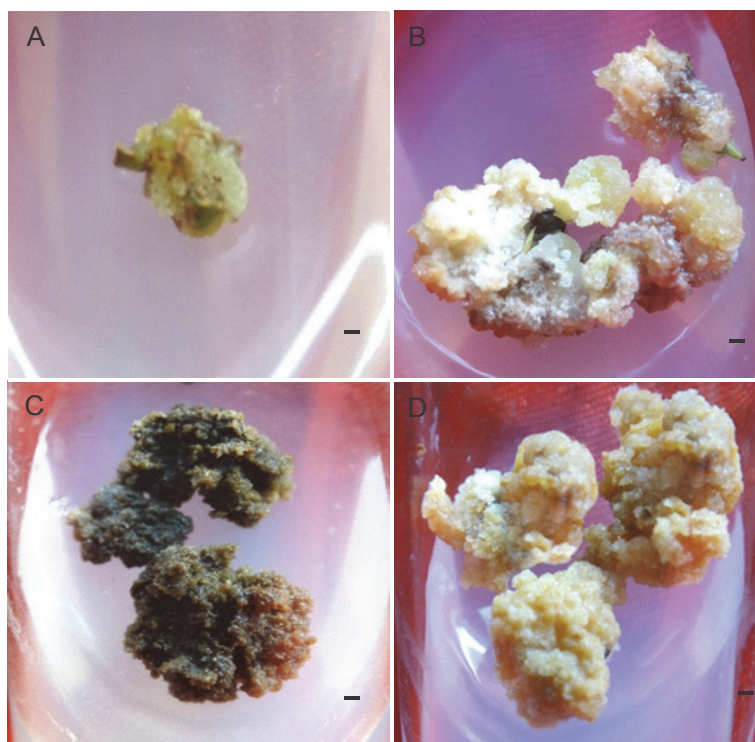

Fig. 1. Callus induced from a stem explant; the medium was amended with 2.0 mg/l 2,4-D: A) early induced callus and B) proliferated callus at the later stage, C) brown callus, and D) callus grown on ascorbic acid-added medium (scale bar, A–D: 0.5 cm)
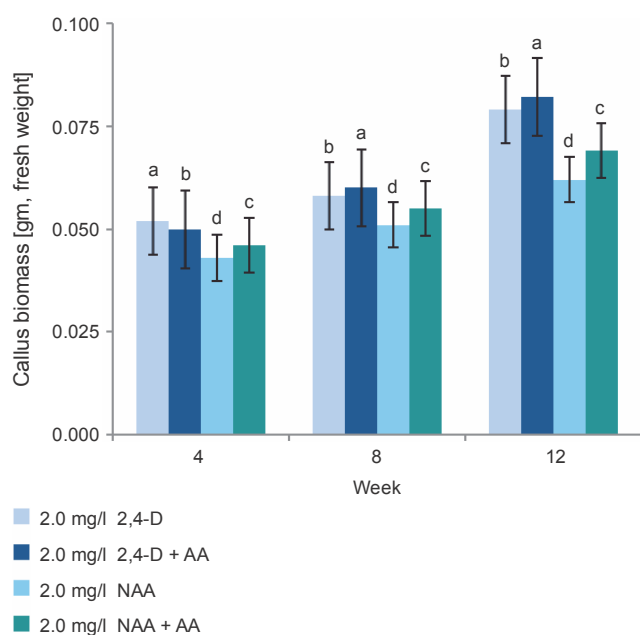
Fig. 2. Callus biomass growth on 2,4-D and NAA amended medium with or without ascorbic acid (AA); values are mean ± standard error of 3 replicates; within each bar, mean values followed by a letter are not significantly different at $P \le 0.05$ according to DMRT
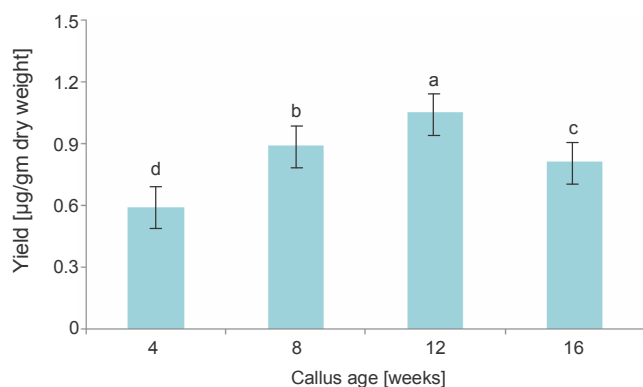

Fig. 3. Alkaloid yield in stem callus cultivated in 2,4-D-amended MS medium for varying periods; values are mean ± standard error of three replicates; within each bar, mean values followed by a letter are not significantly different at $P \le 0.05$ according to DMRT

SOD and APX activities and proline content

The callus grown in the presence of auxin, particularly on 2,4-D, showed an increased level of taxol (1.053 µg/g dry weight) when compared with that cultivated in the control, i.e. without auxin (0.672 µg/g dry weight) (Table 2). As the external auxin supply is considered to be a stressor in cultivated tissues, to analyse the level of stress in auxin- and nonauxin-amended tis-

Table 2. Alkaloid yield in *in vitro* cultivated stem-derived callus and *in vivo* grown tissues; MS medium was added with various PGRs at the concentration of 2.0 mg/l

PGR [mg/l]	Yield [µg/g dry weight]	Yield increase [%]
Control	0.672 ± 0.023 [d]	NA
2,4-D (2.0)	1.053 ± 0.023 [a]	56.69 ± 2.66 [a]
NAA (2.0)	0.896 ± 0.018 [b]	33.33 ± 2.21 [b]
BAP (2.0)	0.696 ± 0.022 [d]	03.57 ± 0.23 [c]
Leaf (*in vivo*)	0.317 ± 0.028 [e]	NA
Stem (*in vivo*)	0.688 ± 0.016 [c]	NA

The values are expressed as mean ± standard error of three replicates of two experiments; within the column, values followed by a different letter are significantly different at $P \le 0.05$ according to the Duncan's Multiple Range Test; NA – not applicable as the values are from *in vivo* grown plant parts and compared with the controls; the controls represent the callus cultured on a PGR-free medium

sues, the activities of the antioxidant enzymes SOD and APX and the level of proline were studied (Table 3). The callus tissues grown on 2,4-D-supplemented medium showed higher SOD activity (3.91 EU min$^{-1} \cdot$ mg^{-1} protein) than the callus (2.58 EU min$^{-1} \cdot$ mg^{-1} protein) cultured on the medium devoid of PGRs. The SOD activity was also high (3.01 EU min$^{-1} \cdot$ mg^{-1} protein) in the callus grown on NAA-supplemented medium. The SOD activity was, however, very low (2.68 EU min$^{-1} \cdot$ mg^{-1} protein), almost equal to the control level, in calli grown on BAP-amended medium. Like SOD, APX also showed a similar pattern; the APX activity was high in callus grown in 2,4-D-supplemented medium (1.61 EU min$^{-1} \cdot$ mg^{-1} protein) when compared with that in the control tissue (0.94 EU min$^{-1} \cdot$ mg^{-1} protein). Furthermore, the APX activity was also higher in NAA-amended medium than in control (1.14 EU min$^{-1} \cdot$ mg^{-1} protein). The APX activity was, however, low in callus grown in BAP-supplemented medium (1.02 EU min$^{-1} \cdot$ mg^{-1} protein), but it was still higher than that of control (0.94 EU min$^{-1} \cdot$ mg^{-1} protein). The level of proline, another stress marker, was found to be high and maximum (6.57 mg g^{-1}) in callus grown in 2,4-D-supplemented medium when compared with that in the control tissue (5.62 mg/g); the proline level decreased in callus grown in NAA-supplemented medium (5.90 mg/g), and its accumulation was minimal (5.27 mg/g) in the callus cultivated in BAP-added medium.
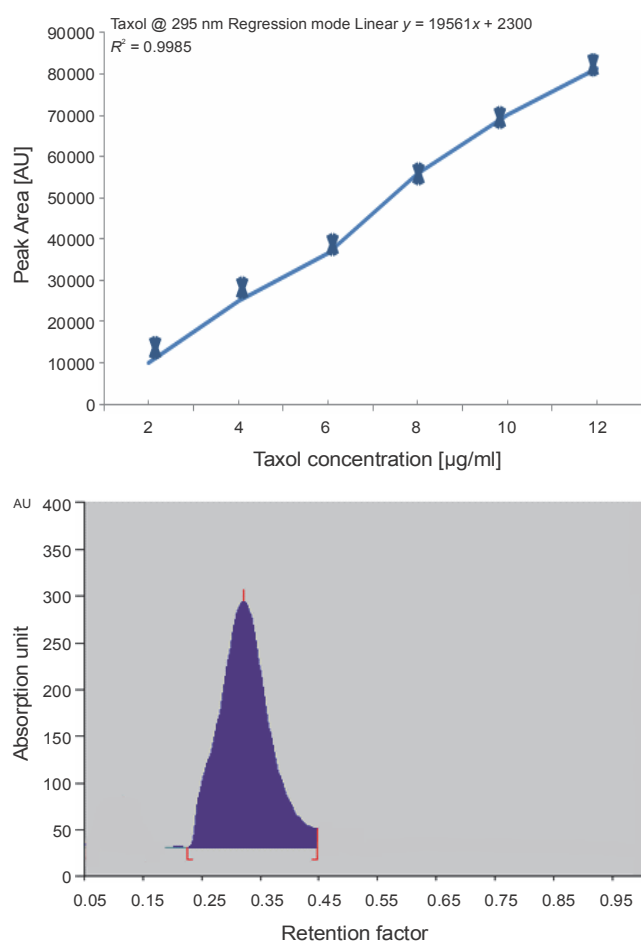
Fig. 4. Standard curve (top) and Peak of Taxol (bottom)

Discussion

Explants, PGRs and callus induction

The present study describes the analysis of the taxol yield in the callus of *T. wallichiana*, which was measured by the HPTLC method. Three different explant types, namely leaf, cone and stem, were tested for their ability to produce callus, and a varied response in callus development was noted. This differential behaviour of explants in influencing morphogenetic responses was reported previously in *Labisia pumila* (Ling et al., 2013) and may be due to the varying levels of endogenous PGRs that cause the explants to behave differently (Ghosh et al., 2014). The exogenous application of auxin, especially 2,4-D, also played a very crucial role in obtaining callus when compared with another tested auxin–NAA; BAP, on the other hand, had a poor influence on the development of callus in *T. wallichiana*. The auxin 2,4-D is usually considered to be the most important trigger in cell division that facilitates the process of callus development (Pasternak et al., 2002; Feher, 2015). In the present study, two auxins were used with or without AA. AA prevents callus browning and improves callus growth by reducing oxidative stress and preventing the oxidation of phenolic compounds (Jones and Saxena, 2013). Treatment with AA (with 2,4-D) was observed to be effective in accelerating callus biomass growth when compared with other auxin treatments.

Callus biomass and taxol yield

From the results of various studies, it is clear that optimization of the medium composition and the composition and concentration of PGRs enables to improve biomass growth in *in vitro* cultures, thus giving the possibility of increasing the levels of therapeutically important phytocompounds (Murthy et al., 2014). The present study clearly indicated that taxol was produced in calli of *T. wallichiana* and the highest yield was observed on 2,4-D-supplemented medium. The study results are very consistent with earlier reports on *Securinega suffruticosa* and *Phyllanthus glaucus* where the biosynthesis of alkaloids was influenced by PGRs and their concentrations (Raj et al. 2015; Sparzak-Stefanowska et al., 2018). It is also confirmed that 2,4-D (1–2 mg/l or more) induced stress in cultured plant cells of *Arabidopsis* and other nonmodel plants (Grossmann, 2000; Feher, 2015) and improved alkaloid yield in *in vitro* grown plant materials. In the present study, in NAA-amended medium, a good taxol yield was obtained and that enriched level may be due to NAA-induced stress or NAA-influenced genes which control the pathways of taxol synthesis. The present study showed that the cytokinin BAP had little influence on alkaloid biosynthesis because the taxol yield was low in the callus and comparable to the control level.

In addition to callus or suspension, the culture of other plant parts (shoot, node etc.) is practised for extracting active compounds (Karuppusamy, 2009; Siahsar et al., 2011), and an overproduction of phytocompounds has been noted in different organs such as shoots, embryos, and protoplast derived from tissues and plantlets (Vinterhalter et al., 2008; Verma et al., 2012; Murthy et al., 2014: Maqsood and Mujib, 2017). In our present study, the callus of *Taxus* was used to measure the level of alkaloids, and it was observed that the yield of taxol gradually increased with callus age. The maximum yield

Table 3. Superoxide dismutase, ascorbate peroxidase and proline levels
in the callus cultured in a medium with and without (control) PGRs [mg/l]

PGR [mg/l]	SOD [min$^{-1} \cdot$ mg^{-1} protein]	APX [min$^{-1} \cdot$ mg^{-1} protein]	Proline [mg/g]
Control	2.58 ± 0.81 [c]	0.94 ± 0.02 [d]	5.12 ± 1.01 [d]
2,4-D 2.0	3.91 ± 0.89 [a]	1.61 ± 0.11 [a]	6.57 ± 1.75 [a]
NAA 2.0	3.01 ± 0.77 [b]	1.24 ± 0.97 [b]	5.90 ± 0.98 [b]
BAP (2.0)	2.68 ± 0.56 [c]	1.02 ± 0.56 [c]	5.27 ± 1.16 [c]

Values are expressed as mean ± standard error of three replicates of two experiments; within the column, values followed by a different letter are significantly different at $P \le 0.05$ according to the Duncan's Multiple Range Test
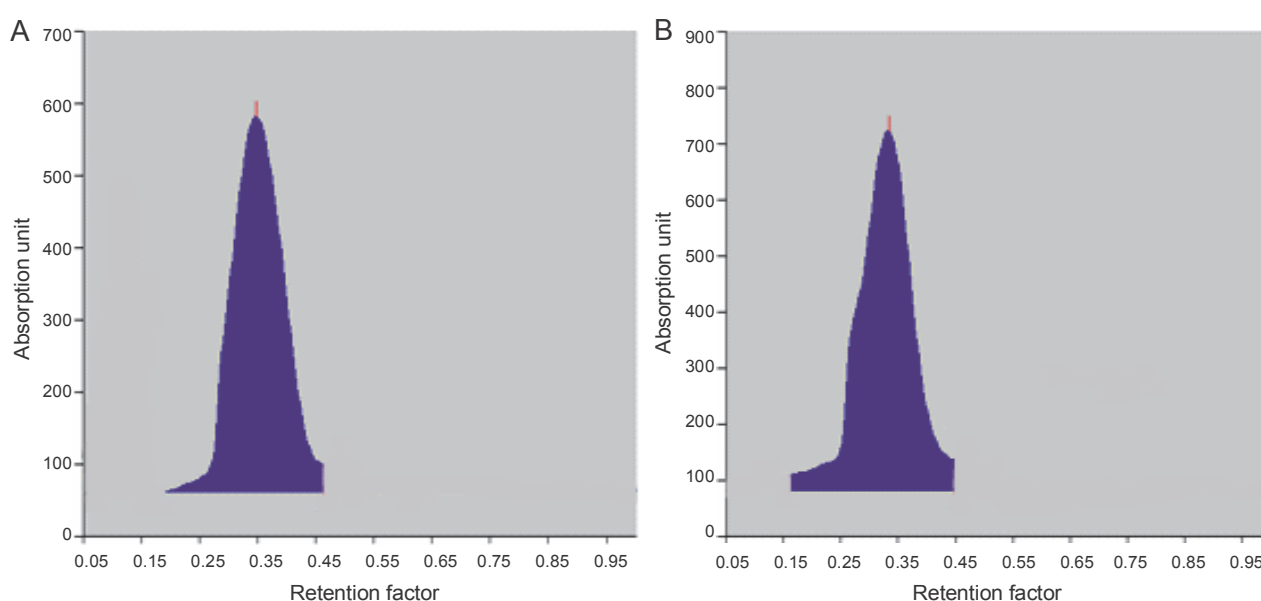

Fig. 5. A) Taxol peak, callus harvested from 2.0 mg/l NAA added medium, and B) Taxol peak, callus was cultivated in 2.0 mg/l 2,4-D added medium

was obtained in 12-week-old callus, and the accumulation then declined with time. Therefore, taxol synthesis was found to be age-dependent. A similar callus age-dependent synthesis was reported in other investigated plant genera such as *Corylus* and *Catharanthus* (Bestoso et al., 2006; Junaid et al., 2010; Almagro et al., 2015).

Antioxidant enzymes and proline as stress markers in response to PGRs

In the present study, it was observed that the yield of taxol was relatively high in the callus grown in 2,4-D-supplemented medium. It is known that 2,4-D induces stress in cultured plant cells (Feher, 2015); thus, some stress markers such as activities of antioxidant enzymes and proline levels were measured in callus tissues sup-

plemented with PGRs. The SOD and APX activities were the highest in the callus grown on 2,4-D-supplemented medium, followed by that in the callus harvested from NAA-amended medium. This enhanced antioxidant enzyme activity in response to various stresses was noted in embryogenic and suspended cells of *Catharanthus roseus* (Elkahoui et al., 2005; Samar et al., 2011; Samar et al., 2015). Pazmino et al. (2011) noted that 2,4-D induced overproduction of reactive oxygen species (ROS) with an increased activation of various antioxidative enzymes in pea plants. In the present study, an increased level of proline was noted in tissues amended with 2,4-D, which suggests the generation of stress; furthermore, in the callus grown in an elevated stress level, a higher level of taxol was recorded when compared with that

grown in less stressful conditions. A higher level of proline accumulation in response to stresses was also observed in several plants such as *Arabidopsis* and *Rauvolfia* (Szabados and Savoure, 2010; Liang et al., 2013; Nadia et al., 2017). A higher accumulation of proline in stressful conditions was earlier reported to be caused by an enhanced expression of the *P5C6* gene, which is associated with proline synthesis (Chen et al., 2009). In rice, DNA methylation modification has been reported to facilitate proline accumulation by upregulating the expression of important genes (Zhang et al., 2013). In addition to the overaccumulation of proline and increased antioxidant enzyme activities, the *Salt Overly Sensitive 1* (*SOS1*) gene has been suggested to be involved in countering stress in plant species of *Centaurium* (Misic et al., 2012). Transcriptional analyses suggested that transcriptional factors such as AP2/ERF and hormone metabolism genes were upregulated by 2,4-D in *Citrus* fruit plant (Ma et al., 2014). Genes encoding stress and defence/pathogenesis-related proteins such as PrP4A, HSP71.1 and HSP71.2 were also similarly activated in pea plant by the exogenous application of 2,4-D (Pazmino et al., 2012). Secondary metabolism genes, particularly those participating in the biosynthesis of lignin and flavonoids, were also noted to be activated significantly in wheat and pea plant materials (Pasquer et al., 2006; Ma et al., 2014). In the present study, the addition of 2,4-D induced stress in *T. wallichiana* callus and improved the alkaloid yield in *in vitro* culture. Thus, supplementation of medium with 2,4-D or an auxin could be a good approach to increase the level of alkaloids in other medicinal plants and could be used on a large scale.

Conclusions

Callus induction, biomass growth, and taxol yield were investigated in *T. wallichiana* under the influence of 2,4-D, NAA and BAP. In media supplemented with 2,4-D and NAA, the callus induction frequency was high, and the callus grew moderately and with less browning when AA was added to the medium. The establishment of callus in a solid and a liquid medium is quite simple, and the use of fermenter/bioreactor may augment the biomass yield further. 2,4-D-supplemented callus tissue showed comparatively high level of taxol. Thus, we believe that the synthesis of taxol may be influenced by PGRs such as 2,4-D in *T. wallichiana* callus. Biochemical

attributes have been presented in support of stress-related outcomes and in taxol synthesis process.

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