Plant Growth Regul (2007) 51:207–215
DOI 10.1007/s10725-006-9161-z

ORIGINAL PAPER

GA_3 induced changes in slow growing endangered Himalayan plant *Podophyllum hexandrum* and hastening of vegetative growth

Rekha Kushwaha · Subedar Pandey · Sanjoy Chanda · Amita Bhattacharya · Paramvir Singh Ahuja

Received: 17 October 2006 / Accepted: 11 December 2006 / Published online: 1 February 2007

Abstract The endangered Himalayan plant *Podophyllum hexandrum* syn. *P. emodi* is inherently slow growing. The plants exhibit delayed emergence of functional leaves or hypocotyl dormancy. However, on GA_3 treatment the functional leaves were found to emerge at a favorable temperature of 25°C in a higher percentage of seedlings and in a shorter time. Functional leaves emerged even at 10°C, a temperature when hypocotyl dormancy generally prevails. A considerable increase in the biochemical parameters related to carbon and nitrogen metabolism [starch, sugars and soluble nitrates, α-amylase and nitrate reductase (NR) activity], respiration and total dehydrogenase activity in all the seedling parts also indicated an enhancement of metabolic processes as influenced by GA_3, for further growth and development. Specific leaf area of the green cotyledonary leaves increased at 25°C, probably to meet the carbon and nitrogen requirements for new structure formation. Higher activity of enzymes involved in carbon and nitrogen metabolism, i.e., NR and α-amylase especially at Hbn, the region of leaf meristematic activity, was further indicative of higher metabolism for earlier initiation of rapid vegetative growth. Initiation of reserve accumulation was also observed at 25°C.

Keywords Biochemical parameters · Functional/true leaf emergence · GA_3 · *Podophyllum hexandrum* · Slow growth · Temperature

Abbreviations

DNS	3,5-Dinitro salicylic acid
LMD	Leaf mass density
LMR	Leaf mass ratio
NEED	*N*-(1-Napthyl) ethylene-diamine dihydrochloride
NR	Nitrate reductase
RS	Reducing sugar
RGR	Relative growth rate
RMR	Root mass ratio
SMR	Shoot mass ratio
SLA	Specific leaf area
TDH	Total dehydrogenase

IHBT Communication No. 508b

R. Kushwaha · S. Pandey · S. Chanda · A. Bhattacharya (✉) · P. S. Ahuja
Division of Biotechnology, Institute of Himalayan Bioresource Technology, Palampur 176 061, Himachal Pradesh, India
e-mail: amitabhatta@yahoo.co.uk

Introduction

Within the rich biodiversity of the Himalayas (Olsen and Larsen 2003) there are rare plant species, many of which are valued for their unique

medicinal properties (Singh and Hajara 1996). *Podophyllum hexandrum*, syn. *P. emodi* is one such plant (Fujii 1991) that is highly sought after by the pharmaceutical industry for its anti-cancer, anti-fungal and immuno-modulatory properties (Kamil and Dewick 1986). The roots and rhizomes of these plants are rich in podophyllotoxins and lignans—the starting material for chemotherapeutic agents like etoposide, teniposide and etopophos (Staheliin and Von Warburg 1991). These are also the approved drugs for the treatment of lung and testicular cancers, leukemias (Staheliin and Von Warburg 1991), rheumatoid arthritis (Lerndal and Svensson 2000), psoriasis and malaria. Consequently, the exploitation of *P. hexandrum* has been ruthless, such that it is now enlisted as an 'endangered plant species' (Nayar and Sastry 1990). Adding to the problem is the extremely slow process of natural regeneration. This slow replenishment of natural populations hardly compensates for the loss of valuable germplasm due to human exploitation.

Slow growth is an adaptive mechanism of most alpine plants for combating the extremely unpredictable and adverse conditions of their natural habitats (Körner 2003) and *P. hexandrum* is no exception. Having to survive the frequent but sudden snow falls and the extreme drought conditions associated with freezing temperatures, the seedlings of *P. hexandrum* exhibit prolonged hypocotyl dormancy followed by senescence of aerial parts. As a result vegetative growth is delayed until the following favorable season of growth (Purohit and Nautiyal 1986). However, since GA_3 is known to hasten vegetative growth in plants (Maksymowych et al. 1976) including *P. hexandrum* (Kharkwal et al. 2004), an attempt was made in the present study to understand the histological and biochemical changes that were affected by GA_3 in order to bring about this hastening of vegetative growth under ex situ conditions. Moreover, since temperature has been found to affect seedling behavior in *P. hexandrum*, attempts were also made to study the interaction of both GA_3 and temperature. In this regard, the biomass allocation as influenced by GA_3 in parameters like leaf mass density (LMD), leaf mass ratio (LMR), shoot mass ratio (SMR) and root mass ratio (RMR), respiration, carbon–nitrogen metabolism and also specific leaf area (SLA) of cotyledonary leaves were studied during seedling establishment. Since the cotyledonary leaves appear to sustain the growth and development of the seedlings during the state of hypocotyl dormancy, the SLA or the key driver of relative growth rate was studied in them. SLA is also known to reflect the amount of carbon invested per unit area of a photosynthesizing leaf in a particular environment.

Materials and methods

Seed collection

Podophyllum hexandrum seeds were collected in August, 2003, 2004 and 2005 from the alpine zone of Koksar (32°22′21″ N; 77°14′05″ E; 3,350 msl) located in the upper parts of the Chandra river valley of the Western Himalayan region (Himachal Pradesh), India. While the maximum temperature of this region ranged between 25 and 30.7°C during the warmer months (July to September), the temperatures during winter months (November to April) was 4–10°C or even less. The temperature at the onset of winter was 4–10°C (November) and that during the onset of summer (May to June), when the snow melted, was 10–18°C. The photoperiod generally ranged between 11 and 13 h with rainfall of 3–4 cm, recorded only on a few days annually.

Seed germination and seedling establishment

Based on the results of our earlier studies, seeds were germinated in plastic pots filled with moist sand in dark in an incubator at 20°C. Five days after germination, the seedlings were transferred to a polyhouse where the temperatures were representative of favorable (25 ± 2°C), unfavorable (4°C) and transitory, i.e., the temperature prevailing during the onset of summer (10°C). The RH (65%) and photoperiod of 12 h light and dark were also maintained. GA_3 at 200 mg l^{-1} (based on earlier observations) was applied to the fully expanded blades of cotyledonary leaves of the seedlings during their transfer to the polyhouse. The untreated seedlings served as the

control and the day of GA_3 treatment was considered as day zero.

Besides percent germination, observations on percentage of seedlings with normal leaf emergence and the time taken for emergence were also recorded. Three replicates with 100 seeds per replicate were taken for each of the treatments and the control.

Evaluation SLA and biomass allocation

Specific leaf area was determined in both GA_3-treated and untreated seedlings by drying cotyledonary-leaf discs from ten different plants (ten discs per replication) at 70°C until a constant mass was obtained (Atkins et al. 1996). The average values for the biomass partitioning parameters (LMRs, SMRs and RMRs) were calculated by dividing dry mass of individual plant part by total plant dry mass, the LMD was determined as the ratio of leaf fresh mass to dry mass.

Biochemical parameters

The seedling parts such as cotyledonary leaves, node at the hypocotyl (Hbn) (Fig. 1) and roots were analyzed for different biochemical parameters.

Starch was estimated in 25 mg of dried tissues of each of the above mentioned seedling parts, each year. Anthrone reagent was used to measure starch as liberated glucose following hydrolysis of the powders (Adams et al. 1980). The 3,5-dinitro salicylic acid (DNS) method of Miller (1959) was used to estimate reducing sugar (RS) in the dried tissue samples.

For α-amylase activity (Bernfield 1955), 100 mg of plant material extracted overnight in chilled 10 mM $CaCl_2$ were centrifuged at 54,000*g* for 20 min at 4°C. Enzyme activity of 100 µl supernatant (enzyme source) was determined by adding 1% starch solution (100 µl) followed by incubation at 30°C for 30 min. DNS reagent (200 µl) was used to stop the reaction by heating in a boiling water bath for 8 min. While hot, 100 µl solution of potassium sodium tartrate was added. Measurement of absorbance at 560 nm on cooling, expressed the enzyme activity as µg of maltose min^{-1} of incubation with 1% starch. For the control, the reaction was terminated at zero time.

Soluble nitrates were estimated in 500 mg of oven-dried samples homogenized in 10 ml of deionized water with a pinch of charcoal and filtered through Whatman No. 1 filter paper. To aliquots dried in vacuo, 3 ml of phenol-di-sulfonic acid

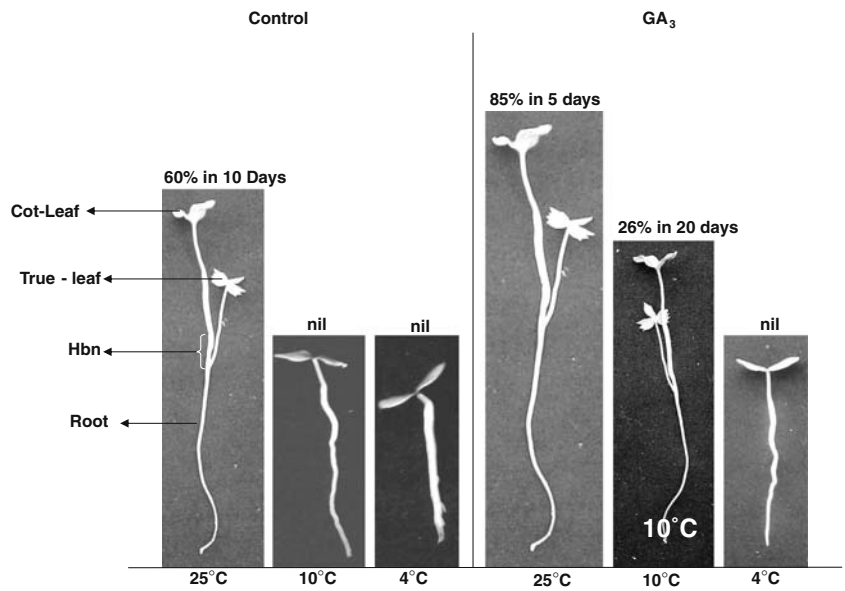

Fig. 1 Photographs representing true leaf emergence in *P. hexandrum* grown at different temperatures with and without GA_3 treatment. *Values* above the bars indicate % and days of true leaf emergence. Different parts of *P. hexandrum* seedlings showing initiation of true leaf emergence have been designated

was added and incubated for 10 min. To this, 15 ml de-ionized water was added along with 1:1 ammonia solution until an alkaline pH was attained. The volume was made up to 100 ml with de-ionized water and the absorbance was measured at 420 nm.

Nitrate reductase (NR) activity was determined by vacuum-infiltrating 25 mg plant tissues in 1 ml of assay solution [100 mM phosphate buffer (pH 7.5), 1.5% (w/v) KNO_3 and 1.5% (v/v) *N*-propanol] for 5 min. The tissues were incubated in darkness at 28°C for 2 h and filtered. 0.5 ml of the assay mixture thus obtained was then mixed with 1 ml of color reagent [1% sulfanilic acid and 0.02% *N*-(1-napthyl) ethylene-diamine dihydrochloride] in the ratio of 1:1 and incubated for 10 min. The absorbance was read at 540 nm (Stewart and Orebamjo 1979) and NR activity was determined using a standard curve prepared from KNO_2, and expressed as mmol NO_2 g^{-1} dry weight h^{-1}.

Respiration was polarographically measured using a computerized Hansatech (UK) oxygen electrode in terms of oxygen taken up by plant samples suspended in air-saturated water for 10 min in the dark (Pandey et al. 1998). For total dehydrogenase (TDH) activity, the plant samples were infiltrated with 2,3,5-triphenyl tetrazolium chloride (5% w/v in 50 mM phosphate buffer, pH 7.5) in a vacuum and incubated for 24 h at 28°C. The formazan formed was extracted in ethanol and the absorbance was read at 510 nm.

Histological studies

The GA_3-treated seedlings growing at the three selected temperatures (4, 10 and 25°C) were subjected to anatomical studies after a week. The node at the hypocotyl (about 5 mm in length) from these plants was fixed in FAA (Formalin, acetic acid and 50% ethyl alcohol: 1:1:18) for 5 days. The samples were dehydrated in a tertiary butyl alcohol series followed by infiltration by paraffin wax (m.pt. 56–58°C) and finally embedded in paraffin wax blocks. Sections, 10 μm thick, were cut with the help of microtome (Shandon Finesse ME, Thermo Electron Corporation). Gelatin jelly (1%) was used as an adhesive. The slides with the sections were stretched on a hot plate at 50°C followed by staining in Safranin and Fast Green and mounted in DPX [80–10 g Distrene (British resin product), 5 cm^3 dibutyl phthalate and 35 cm^3 xylene]. The photographs were taken under a microscope Nikon (Biophot) No. 78508 (Japan) at 40× magnification using a digital camera (Nikon DXM 1200).

Statistical analysis

Standard deviation was calculated from ten replicates, for each of the parameters studied.

Results

Seed germination and seedling establishment

About 90–92% seed germination was observed within 20 days in *P. emodi*. When the 5-day-old seedlings were transferred to (25 ± 2°C) under polyhouse condition, they grew vigorously into healthy seedlings with fully expanded cotyledonary leaves within a week. The time taken for the emergence of true or functional leaves in the untreated control was 10 days and 60% of the seedlings showed true leaf emergence (Fig. 1). However, a reduction in the time taken to leaf emergence in an increased number of seedlings was observed after GA_3 treatment. Thus, 85% of the seedlings that were treated with GA_3 showed true leaf emergence within a span of only 5 days (Fig. 1). The growth of the control seedlings (untreated) at 4 and 10°C was poor and no leaf emergence was observed in these even after 30 days (Fig. 1). Rather, the ones growing continuously at 4°C showed signs of senescence after about 10–15 days. However, the senescence was delayed by 2 weeks in the GA_3-treated seedlings growing at 4°C and leaf emergence was observed in 26% of the seedlings at 10°C after 20 days.

SLA, LMD and biomass allocation

Although GA_3 treatment did not show much effect on LMD, LMR, SMR or RMR of seedlings,

Table 1 Changes in specific leaf area (SLA), leaf mass density (LMD), biomass allocation pattern and true leaf emergence of *P. hexandrum* seedlings grown at different temperatures (4, 10, 25°C) with and without GA_3 treatment (± values represents std deviation)

Physio-biochemical parameters	Control			GA_3 (200 mg l^{-1})		
	4°C	10°C	25°C	4°C	10°C	25°C
SLA (m^2 kg^{-1})	141 ± 11.0	146 ± 10	167 ± 10	141 ± 9	158.9 ± 8	179.6 ± 11
LMD	8.5 ± 1.6	8.21 ± 1.6	6.85 ± 1.2	8.4 ± 2.3	6.2 ± 2.4	5.8 ± 2.5
Leaf mass ratio	0.48 ± 0.2	0.51 ± 0.02	0.51 ± 0.2	0.48 ± 0.08	0.51 ± 0.05	0.53 ± 0.45
Shoot mass ratio	0.29 ± 0.09	0.25 ± 0.1	0.26 ± 0.023	0.29 ± 0.08	0.26 ± 0.06	0.25 ± 0.09
Root mass ratio	0.23 ± 0.07	0.24 ± 0.09	0.23 ± 0.08	0.23 ± 0.06	0.23 ± 0.07	0.22 ± 0.09
True leaf emergence (%)	–	–	60 ± 1.9	–	26 ± 2.5	85 ± 4.1

yet SLA of the cotyledonary leaves was significantly enhanced (Table 1).

Biochemical parameters

GA_3 was found to influence the biochemical parameters, irrespective of the temperatures at which the seedlings were grown. Differences between the temperatures in both the treated and control seedlings were also observed (Table 2). At all the temperatures tested, GA_3 increased the starch content in the seedling parts (cot leaf, Hbn and roots) with respect to their respective counterparts in the control (Table 2). Furthermore, higher contents of starch were recorded in the seedlings placed at 10°C as compared to those at 4°C (Table 2). Between the seedling parts, the highest starch in roots of seedlings placed at 25°C as compared to a decline in the other seedling parts (Hbn and cot leaf) was recorded (Table 2). Albeit lower, a trend similar to that of GA_3-treated seedlings was observed in the untreated control at all temperatures and seedling parts. The only exception was observed in the roots of seedling growing at 25°C (Table 2).

The content of RS and α-amylase activity in plant parts of both the GA_3-treated and control seedlings growing at the different tested temperatures, increased or decreased proportionately. Generally, whenever, the starch content increased or decreased, the α-amylase activity and RS also increased or decreased accordingly (Table 2). An overall increase in starch, RS and α-amylase activity was observed after GA_3 treatment, as compared to the seedling parts of control at different temperatures (Table 2). However, the α-amylase activity was found to decrease in the roots of the GA_3-treated seedlings grown at 25°C.

In all seedling parts of GA_3-treated seedlings and roots of control, nitrate was found to increase with increase in temperature (Table 2). Nitrate and NR activity was higher in the treated seedlings as compared to their control counterparts. The only exception was observed in cotyledonary-leaf of GA_3-treated seedlings placed at 10°C where nitrate content was less than in the control (Table 2).

When the respiration and TDH activity was measured in the different seedling parts growing at the tested temperatures, it was found to increase with increasing temperatures. Considerable increase in these was also observed after GA_3 treatment (Table 2).

Histological studies

The pattern of development of leaf primordia varied greatly in the seedlings maintained at the three temperatures studied. While the development of leaf primordia in order to achieve the rosette characteristic was rapid at 25°C in the control i.e. within 10 days (Fig. 1), that in the seedlings growing at 10°C was slow (Fig. 1). Development of leaf primordia (Fig. 2) was the slowest and poorest in the seedlings growing at 4°C, even after 30 days. On application of GA_3, development of leaf primordia enhanced and the time for emergence of true leaves was also found to be reduced (5 days). It was surprising that on application of GA_3, true leaf development was also enhanced at 10°C and true leaf emerged within 20 days (Fig. 2).

Table 2 Changes in various biochemical parameters in root, Hbn and cot leaf during functional leaf emergence in *P. hexandrum* seedlings grown at different temperatures (4, 10 and 25°C) with and without GA_3 treatment (± values represents std deviation)

Biochemical parameters		Control			GA_3 treated		
		4°C	10°C	25°C	4°C	10°C	25°C
Starch (mg g^{-1} dry mass)	Root	0.51 ± 0.06	1.37 ± 0.04	1.35 ± 0.23	0.82 ± 0.45	1.78 ± 0.23	2.96 ± 0.2
	Hbn	1.42 ± 0.4	1.51 ± 0.05	1.36 ± 0.76	0.43 ± 0.34	2.06 ± 0.23	1.19 ± 0.4
	Cot leaf	0.42 ± 0.3	1.31 ± 0.2	1.03 ± 0.23	0.73 ± 0.45	1.94 ± 0.34	1.08 ± 0.3
Reducing sugar (mg g^{-1} dry mass)	Root	0.053 ± 0.006	0.15 ± 0.34	0.06 ± 0.04	0.09 ± 0.03	0.30 ± 0.09	0.18 ± 0.8
	Hbn	0.068 ± 0.07	0.03 ± 0.34	0.25 ± 0.04	0.26 ± 0.04	0.29 ± 0.08	0.39 ± 0.7
	Cot leaf	0.03 ± 0.04	0.15 ± 0.56	0.20 ± 0.08	0.05 ± 0.005	0.31 ± 0.078	0.32 ± 0.5
Amylase (μg maltose produced g^{-1} tissue min^{-1})	Root	1.094 ± 0.5	3.77 ± 0.12	3.96 ± 0.45	2.245 ± 0.12	4.21 ± 0.23	2.14 ± 0.56
	Hbn	1.734 ± 1.3	5.7 ± 0.23	3.17 ± 0.34	2.005 ± 0.23	7.9 ± 0.56	3.84 ± 0.89
	Cot leaf	1.76 ± 3.9	4.18 ± 0.34	7.61 ± 0.67	2.9 ± 0.24	7.8 ± 0.45	3.6 ± 0.5
Nitrate (μg g^{-1} dry wt)	Root	34.49 ± 5.9	37.62 ± 3	81.52 ± 4	53.30 ± 5	78.38 ± 5	163.04 ± 9
	Hbn	56.43 ± 7.9	288.46 ± 6	228.88 ± 60	244.56 ± 9	406.60 ± 6	420.15 ± 54
	Cot leaf	689.8 ± 8.0	1,348.24 ± 45	940.63 ± 73	993.93 ± 16	658.45 ± 34	1,191.47 ± 78
Nitrate reductase (μmol NO_2 h^{-1} g^{-1} dry mass)	Root	0.007 ± 0.01	0.006 ± 0.098	0.11 ± 0.02	0.009 ± 0.07	0.022 ± 0.078	0.023 ± 0.08
	Hbn	0.012 ± 0.02	0.047 ± 0.089	0.32 ± 0.03	0.043 ± 0.08	0.11 ± 0.067	0. 59 ± 0.07
	Cot leaf	0.15 ± 0.04	0.22 ± 0.05	0.134 ± 0.04	0.18 ± 0.9	0.191 ± 0.034	0.168 ± 0.06
Respiration (nmol g^{-1} min^{-1})	Root	84.66 ± 4.8	133 ± 13	155 ± 12	100 ± 10	152 ± 12	153 ± 12
	Hbn	93.88 ± 3.8	138.3 ± 4	164 ± 12	120 ± 23	159 ± 9	182 ± 11
	Cot leaf	128.8 ± 7.6	151 ± 10	189 ± 11	151 ± 32	160 ± 10	194 ± 8
Total dehydrogenase activity (μmol formazan produced g^{-1} tissue h^{-1})	Root	0.57 ± 0.02	1.10 ± 0.26	1.40 ± 0.08	0.78 ± 0.1	1.31 ± 0.6	1.40 ± 0.76
	Hbn	0.68 ± 0.06	1.03 ± 0.034	1.50 ± 0.01	0.91 ± 0.7	1.47 ± 0.9	1.59 ± 0.34
	Cot leaf	1.0 ± 0.001	1.52 ± 0.23	1.80 ± 0.08	1.23 ± 0.3	1.32 ± 0.6	1.85 ± 0.45

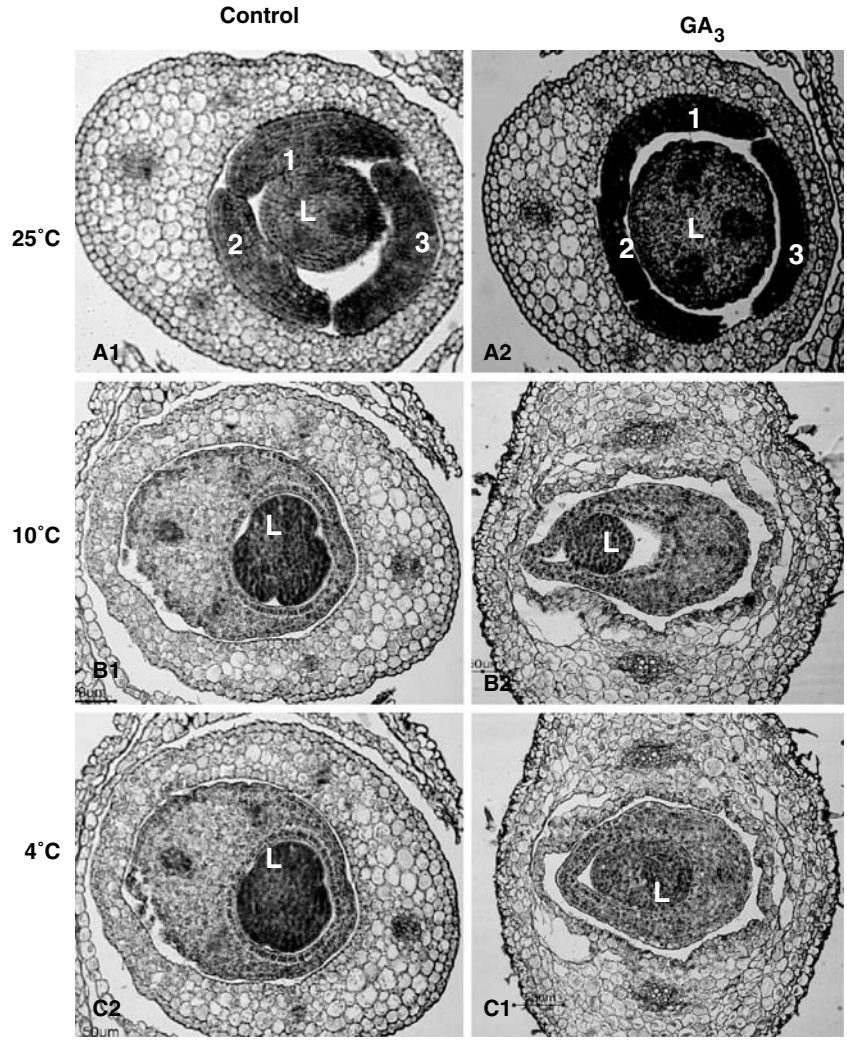

Fig. 2 Transverse sections of the node at hypocotyl (Hbn) showing the ontogeny of functional leaf development in *P. hexandrum* seedlings, in response to different temperatures, with and without GA_3 treatment, i.e., (A1-2) 25°C, (B1-2) 10°C and (C1-2) 4°C (the primordia are numbered in serial order)

Discussion

GA_3 associated ontogenic and biochemical changes during the enhancement of vegetative growth and early emergence of functional leaves in *P. hexandrum* have been discussed in the present paper. *P. hexandrum* is a slow growing species and its growth and reproduction is governed mainly by temperature, besides other factors (Pollock et al. 1983). Slow growth is a characteristic feature of most alpine plants (Atkin and Day 1990) when major metabolic processes are repressed. It is obvious that the metabolism of carbon and nitrogen plays a crucial role during plant growth (Lattanzi et al. 2005). However, these can be influenced at times and such an attempt was made in the present study by employing GA_3 treatment for domesticated plants of *P. hexandrum.* That GA_3 (Emery et al. 2001) and temperature are two regulatory factors directly or indirectly governing growth and various metabolic processes is a well documented fact (Loveys et al. 2002). Thus, functional leaf emergence was observed in a higher percentage of seedlings with increasing temperature and this was further enhanced when GA_3 was used. Even the time taken for leaf emergence was further reduced (5 days) as compared to plants without GA_3 treatment (10 days). Since emergence of functional leaves initiates the process of further vegetative growth in *P. hexandrum*, it is natural that respiration and TDH activity would also

increase as respiration is proportional to the rate at which the new structures are being formed (Amthor 1989). This is also indicative of the fact that more photosynthates would be formed as respiration provides the ATP, reducing equivalents and carbon skeletons required for the synthesis of new structures. True to this fact, increased SLA of the fully expanded green cotyledonary leaves in the GA_3-treated seedlings of *P. hexandrum* (179.6 m^2 kg^{-1}), as compared to control (167 m^2 kg^{-1}), resulted in earlier emergence and growth of functional leaves at 25°C (85% in 5 days). The fully expanded, green cotyledonary leaves probably support the formation of true leaves and hence further vegetative growth. High SLA values are known to maximize the amount of carbon gained per unit leaf dry mass as per available leaf area (Atkins et al. 1996). However, no change in biomass allocation pattern in different seedling parts after GA_3 treatment (Table 1), suggest the utilization of all the extra photosynthates thus produced for the emergence of true leaf rather than storage of assimilates as reserves. Allocation of reserves to different plant parts for the initiation of faster and earlier vegetative growth after GA_3 treatment did occur at favorable temperatures, i.e., 26% after 20 days at 10°C and 85% after 5 days at 25°C (Table 1). This was supported by an overall increase in starch, sugars and soluble nitrates coupled with α-amylase and NR activity (Table 2) in all the seedling parts, since structure formation requires allocation and assimilation of both carbon and nitrogen. A sudden spurt in these parameters at 10°C but a slight decline at 25°C is further indicative of greater utilization of carbon for the synthesis of new structures and rapid growth of existing ones at favorable temperatures. Some accumulation of reserves is also initiated at this time. Thus, α-amylase (Table 2) activity increased in the Hbn, i.e., the region of leaf primordia activity and development, but decreased in roots and cotyledonary-leaf of treated seedlings. On the contrary, starch content in the roots increased considerably. While soluble nitrates and NR activity (Table 2) was maximal in the cotyledonary-leaf, it was lowest in the roots and became more pronounced after GA_3 treatment. From these observations, it appears that the nitrates that were taken up by the roots were probably translocated to the photosynthetic organs (i.e., the cotyledonary-leaf in the present study) as has been observed in many herbaceous plants (Gojon et al. 1994).

Histological studies (Fig. 2) also confirmed the role of GA_3 in enhancement of growth and development of leaf primordia. Fastest development in treated seedlings placed at 25°C and enhancement of leaf primordia development even at the lower temperature, i.e., 10°C, in treated seedlings further proved the role of GA_3 in enhancement of vegetative growth.

Acknowledgement The authors acknowledge the financial assistance obtained from the National Bioresource Development Board, Department of Biotechnology, Government of India, New Delhi.

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