

# Temperature-dependent growth and emergence of functional leaves: an adaptive mechanism in the seedlings of the western Himalayan plant *Podophyllum hexandrum*

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**Abstract** As an adaptive mechanism, hypocotyl dormancy delays emergence of functional leaf until favorable season of growth in *Podophyllum hexandrum*, an endangered medicinal plant of the western Himalayas. However, upon exposure of the freshly germinated seedlings to favorable temperature (25°C), functional leaves emerged within 20 days. Therefore, we examined regulation mechanisms of growth and development of this alpine plant by temperature under laboratory conditions. The seedlings were exposed to (1) 25°C (temperature prevailing at the time of maximum vegetative growth), (2) 4°C (mean temperature at the onset of winter in its natural habitat), and (3) 10°C (an intermediate temperature). Slackened growth at 4°C was followed by senescence of aerial parts and quiescence of roots and predetermined leaf primordia. Rapid development of leaf primordia at 25°C was associated with increased starch hydrolysis. This was evident from higher  $\alpha$ -amylase activity and reducing sugars. These

parameters decreased on sudden exposure to 4°C. In contrast, the roots (perennating organs) showed a slight increase (1.36-fold) in  $\alpha$ -amylase activity. Growth and development in seedlings growing at 10°C (temperature less adverse than 4°C) were comparatively faster. The content of reducing sugars and  $\alpha$ -amylase activity were also higher in all the seedling parts at 10°C as compared to 4°C. This indicated larger requirements for sugar by the seedlings at 10°C. Irrespective of temperature, maximum changes in nitrate and nitrate reductase occurred during the initial 10 days, i.e., when the readily available form of sugars (reducing sugar) was highest. This indicated that a temperature-dependent availability of carbon, but not temperature itself, was an important regulator of uptake and reduction of nitrogen.

**Keywords** Adaptive mechanism · Biochemical parameters · Western Himalayas · Leaf emergence · *Podophyllum hexandrum* · Temperature

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## Introduction

*Podophyllum hexandrum* syn. *P. emodi* Royle is an important high-altitude, shade-loving (Pandey et al. 2006) perennial plant of the western Himalayas (Stahelin and Warburg 1991; Kharkwal et al. 2004). Its roots and rhizomes rich in podophyllotoxins, are highly valued for their anticancer, antimetabolic and immunostimulatory properties (Kaplan 1942; Loike et al. 1978; Pugh et al. 2001). The semisynthetic derivatives of these lignans—the etoposide (VP-16-213), teniposides (VM-26), and etopophos are also the approved drugs for the treatment of testicular cancer, small-cell lung cancer (Stahelin and Warburg 1991) and rheumatoid arthritis (Lerndal and Svensson 2000).

The natural habitats of *P. hexandrum*—the alpine/sub-alpine Himalayan regions are characterized by freezing temperatures, high winds, and short growing season (Ram and Singh 1994). Plants that grow in such alpine climates show a very high degree of specialization for either avoiding or tolerating the alpine environmental conditions (Körner and Spehn 2002; Körner 2003). *P. hexandrum* appears to avoid rather than tolerate the unfavorable alpine winter conditions by employing several adaptive mechanisms. Firstly, the seeds mature and germinate in large numbers during the favorable warm season (20–25°C) only. Secondly, the seedlings that finally establish themselves exhibit “hypocotyl dormancy” or “pseudomonocotily” (Purohit and Nautiyal 1986; Kharkwal et al. 2004). This is a phenomenon wherein the long tubulate parts of the two cotyledonary leaves of the newly emerged seedlings fuse to form a sheathing base that covers the plumule or epicotyl (Terabayashi 1987). This prevents the emergence of functional leaves until the following favorable season of growth (Kharkwal et al. 2004). The cotyledonary leaves expand into healthy green photosynthetic structures and make the best use of the favorable short season of growth. The cotyledonary leaves undergo senescence at the onset of winter when the mean maximum air temperature is 10°C. *P. hexandrum* is survived only by the perennating underground parts at a depth of about 10–12 cm where the mean temperature is about 4°C. Hypocotyl dormancy is broken and vegetative growth resumes from these perennating organs only during the following summer season when temperatures are favorable (20–25°C) again. The functional leaves emerge either during the following (Kharkwal et al. 2004; Terabayashi 1987) or the same season of germination (Terabayashi 1987). The growing periods of the slow-growing alpine plants being short, complete differentiation and full cuticular protection of aerial green parts do not occur (Kacperska 1999).

The hypocotyl dormancy strategy employed by *P. hexandrum* for survival in the alpine Himalayan environment was reported to be regulated by cotyledonary leaves (Purohit and Nautiyal 1986). In a previous observation, we found “hypocotyl dormancy was broken whenever the temperature was favorable (20–25°C) but persisted at 10–15°C or lower”. Since temperature appeared to influence the start and break of hypocotyl dormancy of this shade-loving plant (Kushwaha, personal observation), the response of the newly emerged seedlings to three representative temperatures, i.e., (1) 25°C, the temperature at which hypocotyl dormancy is broken, (2) 10°C, the mean maximum temperature during onset of winter or snow melt or the time when hypocotyl dormancy persists, and (3) 4°C, the mean maximum temperature of early winter and also the mean root zone temperature of perennating organs, was tested under lab conditions.

The overall aim of the present paper was to investigate (1) whether hypocotyl dormancy was exhibited to avoid investing valuable resources in new structures like leaves that would ultimately be lost due to senescence during the adverse temperatures of the oncoming winter, (2) the presence of predetermined meristems and their development for maximum utilization of the following short season of growth after the winter months, and (3) whether the predetermined meristems and perennating organs were maintained during the winter months by carbon and nitrogen metabolism of the seedlings. Starch, reducing sugars (RS), and nitrates in the different seedling parts are supposed to indicate the amount of photosynthates/assimilates that were invested/accumulated or distributed in response to temperature. The relative amounts of the enzymes such as  $\alpha$ -amylase and nitrate reductase coupled with leaf ontogeny, respiration, and TDH were presumed to indicate the utilization of carbon and nitrogen for growth and maintenance. Formation of new structures and their growth and maintenance are known to depend on allocation and assimilation of carbon and nitrogen. Given the pronounced seasonality of *P. hexandrum*, these studies are presumed to provide an insight into the survival strategies employed by these seedlings in the harsh alpine climate. The knowledge gained will be useful for workers interested in ex situ conservation of the plants for sustainable use.

## Materials and methods

*P. 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 m asl) located in the upper parts of the Chandra River valley of the western Himalayan region (Himachal Pradesh) of India.

### Environment at Koksar and behavior of *P. hexandrum* under in situ conditions

Environmental factors including precipitation (i.e., snowfall and rainfall), snow cover, and mean minimum and maximum air temperatures of Koksar were recorded during the different seasons for a proper understanding of the natural habitat of *P. hexandrum*. The soil temperature at the surface and 5 and 12 cm below the surface at special niches where *P. hexandrum* grew were also recorded using a soil thermometer (Mextech, multi-thermometer). All observations were recorded for three consecutive years (2003, 2004, and 2005).

Visual observations of vegetative growth, development and overall response of *P. hexandrum* to different seasons under in situ conditions were recorded each year from 2003 to 2006.

### Seed germination, seedling establishment, and functional leaf emergence under ex situ conditions

Based on the results of our earlier studies, seeds were germinated in plastic pots filled with moist sand in an incubator (B.O.D.) adjusted to 20°C in dark. A week after germination, the seedlings were transferred to three different temperatures representative of the (1) mean temperature at the onset of winter in the plant's natural habitat (4°C), (2) temperature prevailing at the time of maximum vegetative growth (25°C), and (3) an intermediate temperature (10°C) in order to evaluate the effect of these temperatures on further growth and development of *P. hexandrum* seedlings. For further confirmation of the effect of temperature on seedling behavior, the seedlings growing at 4°C were transferred to 25°C, and those at 25°C were transferred to 4°C after 1 month. B.O.D. incubators adjusted to the above temperatures with photoperiods of 11 h light ( $52 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 13 h dark were used for the above experiments.

Observations on percent germination, percentage of seedlings with normal leaf emergence, and the time required to achieve these were recorded for each treatment. Three replicates with 100 seeds per replicate were taken for each of the treatments and control.

### Histological studies for leaf ontogeny

The seedlings growing at the three selected temperatures (4, 10, and 25°C) were subjected to anatomical studies after an interval of 10, 20, and 30 days. The nodes at the base of tubulate cotyledons (about 5 mm in length) were fixed in FAA (formalin, acetic acid and 50% ethyl alcohol: 1:1:18) for 5 days. The samples were dehydrated in tertiary butyl alcohol (TBA) series followed by infiltration in paraffin wax (m. pt. 56–58°C) and finally embedded in paraffin blocks. Sections of 10  $\mu\text{m}$  thickness were cut with the help of microtome (Shandon Finesse ME, Thermo Electron) and sections were fixed on slides using Gelatin jelly (1%). 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<sup>3</sup> 3-dibutylphthalate, and 35 cm<sup>3</sup> xylene]. The photographs were taken under a microscope Nikon (Biophot) no. 78508 (Japan) at 40 $\times$  magnification using a digital camera (Nikon DXM 1200).

### Biochemical parameters

Different seedling parts including (1) cotyledonary leaves (cot-leaf), (2) nodes at the base of tubulate cotyledons (Btc), and (3) roots were used to study the changes in biochemical parameters in response to the three temperatures described

above. The levels of starch, reducing sugars (RS), and nitrate were measured along with the activities of enzymes including total dehydrogenase (TDH), nitrate reductase (NR), and  $\alpha$ -amylase. Samples for biochemical studies were collected at 11:00 a.m.

### Estimation of starch, reducing sugars, and $\alpha$ -amylase activity

About 25 mg fresh tissue of root, cot-leaf, and Btc of the seedlings was used for estimation of starch and RS. Starch was measured as liberated glucose using anthrone reagent following the hydrolysis of the extracted powders with perchloric acid (Adams et al. 1980), whereas RS was measured using the DNS (3,5-dinitro salicylic acid) reagent (Miller 1959). The  $\alpha$ -amylase activity was determined as per the method of Bernfield (1955). About 100 mg of plant material was extracted overnight in chilled 10 mM CaCl<sub>2</sub> solution at 4°C and centrifuged at 54,000 g for 20 min at 4°C. The supernatant (100  $\mu\text{l}$ ) was used as the enzyme source to which 100  $\mu\text{l}$  of 1% starch solution was added followed by incubation at 30°C for 30 min. The reaction was stopped by heating the reaction mix in a boiling water bath for 8 min after the addition of 200  $\mu\text{l}$  DNS reagent. While it was still hot 40% potassium sodium tartrate (100  $\mu\text{l}$ ) solution was added. After cooling, the absorbance was measured at 560 nm, and the activity was expressed as micrograms of maltose produced per minute of incubation with 1% starch. For control, the reaction was terminated at zero time.

### Nitrate and nitrate reductase activity

About 500 mg of oven-dried sample in 10 ml of deionized water was shaken for 30 min with a pinch of charcoal and filtered through Whatman no. 1 filter paper. To aliquots dried in vacuo, 3 ml of phenol–di-sulphonic acid was added. After 10 min, 15 ml deionized water was added along with 1:1 ammonia solution until alkaline pH was attained. A 100-ml volume was made up with deionized water, and absorbance was measured at 420 nm.

For determination of NR activity, 25 mg of plant tissue in 1 ml of assay solution (100 mM phosphate buffer, pH 7.5, 1.5% (w/v) KNO<sub>3</sub> and 1.5% (v/v), *N*-propanol) was vacuum-infiltrated for 5 min until the sample settled at the bottom. The tissues were then incubated in darkness at 28°C for 2 h after which 0.5 ml of the assay mixture was mixed with 1 ml of color reagent [1% sulphanylic acid and 0.02% NEDD (*N*-(1-naphthyl) ethylene-diamine dihydrochloride)] at a ratio of 1:1. The absorbance was read at 540 nm after 10 min (Orebamjo and Stewart 1979), and nitrate reductase activity was determined using a standard curve prepared from KNO<sub>2</sub> and expressed as millimoles of NO<sub>2</sub> per gram of dry weight per hour.

## Respiration and total dehydrogenase activity

For respiration, oxygen uptake of the plant parts was measured polarographically using a computerized Hansatech (UK) oxygen electrode as described by Pandey et al. (1998). Plant samples were suspended in air-saturated water, and oxygen uptake was recorded after a 10-min incubation in dark.

The total dehydrogenase activity was determined in the plant samples as described by Pandey et al. (1998) wherein the tissues were infiltrated with 2,3,5-triphenyl tetrazolium chloride (5% w/v in 50 mM phosphate buffer, pH 7.5) in vacuum and incubated for 24 h at 28°C. The formazan formed was extracted in ethanol, and absorbance was read at 510 nm.

Each experiment was repeated thrice and three replicates were used for each parameter.

## Statistical analysis

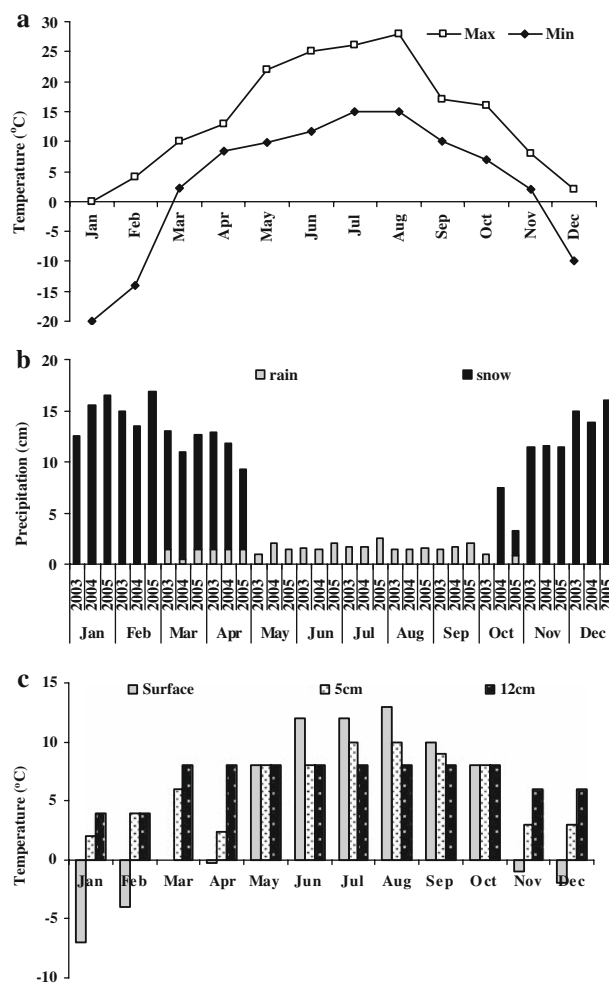
Standard error of means was calculated from 10 replications for each of the parameters studied.

## Results

### Environmental conditions of Koksar

The mean maximum and minimum air temperatures during snow melt or spring (March–May) were 14.5 and 7.4°C, respectively, whereas those during autumn (September–October) were 15.0 and 5.8°C, respectively, and during summer (June–August) were 24.9 and 13.4°C, respectively. Lowest mean maximum and minimum air temperatures were recorded during winter (November–February), i.e., 5.4 and –10°C, respectively. The months of March (snow melt/beginning of summer) and November (beginning of winter) were distinct as the mean maximum air temperature was about 10°C (Fig. 1a). However, since the area belongs to the inner chain of the western Himalayas, the seasonal variations in the growing period were not distinct across the years, and the seasons overlapped and coalesced with each other due to unpredictable rain and snowfall.

Variations in precipitation between the seasons were observed. The mean maximum snowfall (17.4 cm) and rainfall (1.7 cm) were recorded during winter and summer, respectively (Fig. 1b). While the snow failed to persist on the ground during spring, snow during autumn formed a cover of about 6 cm. Thick snow cover of about 45–450 cm was observed in winter when the mean soil temperature at the surface below the snow cover ranged between –2 and –7°C during November–February as compared to 0°C in March. The surface temperature



**Fig. 1** Mean annual environmental data of upper parts of Chandra River valley, Himachal Pradesh, during 2003–2005. **a** Maximum and minimum air temperature. **b** Annual precipitation. **c** Mean soil temperature at the surface and 5 and 12 cm below the surface

continued to increase steadily during April (8°C) to reach a maximum of 13°C during August but declined thereafter from September onwards.

The temperature of the root zone of *P. hexandrum* at a depth of 5 and 12 cm under the snow cover ranged between 2–4°C and 4–6°C during November–February. During March however, the temperature at a depth of 5 cm rose to 6°C, declined again during April (2.4°C) only to increase up to 11°C in August. In contrast, the temperature at 12 cm during March rose to 8°C and remained unchanged until October (Fig. 1c).

### Seed germination and seedling establishment under ex situ conditions

No visible signs of *P. hexandrum* were observed immediately after snow melt (during mid-March) under natural conditions in Koksar. Plants emerged only in the first week

of April. Floral buds developed during the end of April and small green berries formed during the last week of June and first week of July. Berries matured between mid-July and mid-August, dehisced by September, and the seeds germinated within 2–3 weeks provided the temperatures were conducive (20–30°C). The seedlings grew throughout September and October, exhibited hypocotyl dormancy and failed to produce functional leaves. With the advent of winter when the temperatures started dipping to 10°C (i.e., early November), the aerial parts underwent senescence. New shoots emerged from perennating rhizomes only after snow-melt. These observations were recorded only in special pockets/niches where there were no human interventions.

### Seed germination and seedling establishment

After 20 days at 20°C, 90–92% of seeds germinated. Variable responses with respect to functional leaf emergence were observed when healthy seedlings with fully expanded cotyledonary leaves were transferred 1 week after germination at 20°C to the three temperatures stated above. As the cotyledonary leaves shriveled, functional leaves emerged from the node at the Btc within 10 days in the seedlings maintained at 25°C. No emergence of functional leaves was observed in the seedlings transferred to 4 or 10°C even after 30 days. Rather, after 10–20 days of exposure to 4°C, the aerial parts (cotyledonary leaves and the tubulate part) of the seedlings underwent senescence, whereas the underground parts i.e., the Btc, hypocotyls and roots, remained healthy (Fig. 2).

When the seedlings without aerial parts were transferred to 25°C, functional leaves sprouted from the Btc, and the roots grew vigorously (Fig. 2a). On the other hand, the aerial parts of the seedlings that had been growing vigorously at 25°C senesced completely when transferred to 4°C, and their roots became stouter and healthier. However, no change was observed in the Btc (Fig. 2b).

### Ontogenic development of functional leaves

When histological study was carried out to understand the ontogeny of leaf morphogenesis, predetermined leaf meristems were observed in the seedlings irrespective of the temperatures studied (Fig. 3). However, further growth of leaf primordia and leaf emergence were influenced by the temperature of the environment in which the seedlings were growing (Fig. 3). Development of leaf primordia was slowest at 4°C followed by 10°C and then 25°C (Fig. 3b–d).

Although morphogenesis of functional leaves was initiated after 10 days at 4°C (Fig. 3b1), further development occurred only after 20 days (Fig. 3b2) at this temperature. More than one leaf primordium was initiated after 30 days

(Fig. 3b3), and the number increased with time. However, they failed to develop into full-fledged leaves even after 90 days, and there was no functional leaf emergence in seedlings maintained at 4°C (Fig. 3b). On the other hand, both leaf morphogenesis and primordium preformation were considerably hastened at 10°C (Fig. 3c), and as many as 2, 4, or 7 primordia were initiated after 10, 20, and 30 days, respectively (Fig. 3c1–3). Although the primordium preformation in terms of number was more pronounced at this temperature as compared to 4°C, emergence of functional leaves did not occur.

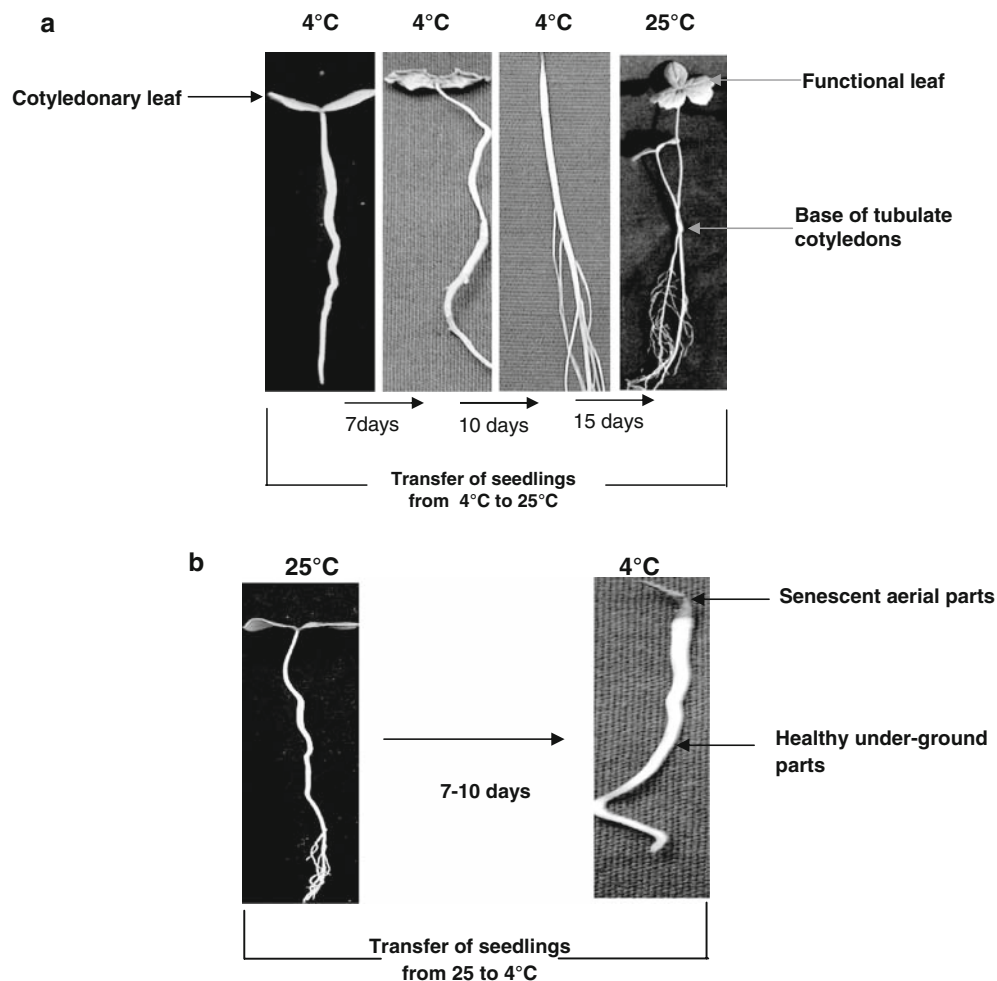
Leaf morphogenesis was fastest at 25°C (Fig. 3d), and the preformation of a number of leaf primordia became evident within 10 days (Fig. 3d1). It also became clear that maximum sets of leaf primordia became distinctly organized at this temperature in a much shorter time. While one set of functional leaves emerged at a particular time, a second set of leaf primordia continued to develop so as to be ready for emergence with time. The development of pairs of leaf primordia appeared to be rather sequential (Fig. 3d1–3). The leaf primordia were protected by the base of the cotyledonary tube, which is composed of a distinct mass of protective tissues probably made of callose (Fig. 3). With further development of the leaf primordia at favorable temperatures (25°C), the protective tissue mass underwent rapid atrophy and was gradually eliminated (Fig. 3). Eventually, the functional leaves emerged. Although atrophy at 4 and 10°C was initiated after 20 (Fig. 3b2) and 10 days (Fig. 3c1), respectively, it was comparatively slower than that at 25°C (Fig. 3d).

### Biochemical parameters

A drastic change in all the parameters in the seedling parts under study was observed during the first 10 days after the seedlings were transferred to the three different temperatures stated above.

Ten days after the seedlings were transferred to the lowest temperature, i.e., 4°C, a decline in starch (Fig. 4a) was coupled with an increase in the activities of enzymes such as  $\alpha$ -amylase (Fig. 4b) and NR (except in the Btc; Fig. 4e) and considerable increase in RS (Fig. 4c) and nitrate, irrespective of the seedling parts under study. All the parameters increased between 10 and 20 days, with the exception of starch (Fig. 4a) in the Btc but decreased with time (20–30 days). After 20 days, decline in all parameters was observed in cot-leaf, Btc (except NR), and root (except  $\alpha$ -amylase). On the other hand, an increase in nitrate was observed in Btc (Fig. 4d).

Ten days after transfer to 10°C, starch had declined (Fig. 4a). A reverse trend was observed in  $\alpha$ -amylase, which increased for 20 days but declined thereafter (Fig. 4b). The nitrate (Fig. 4d) and NR (Fig. 4e) also showed an increase

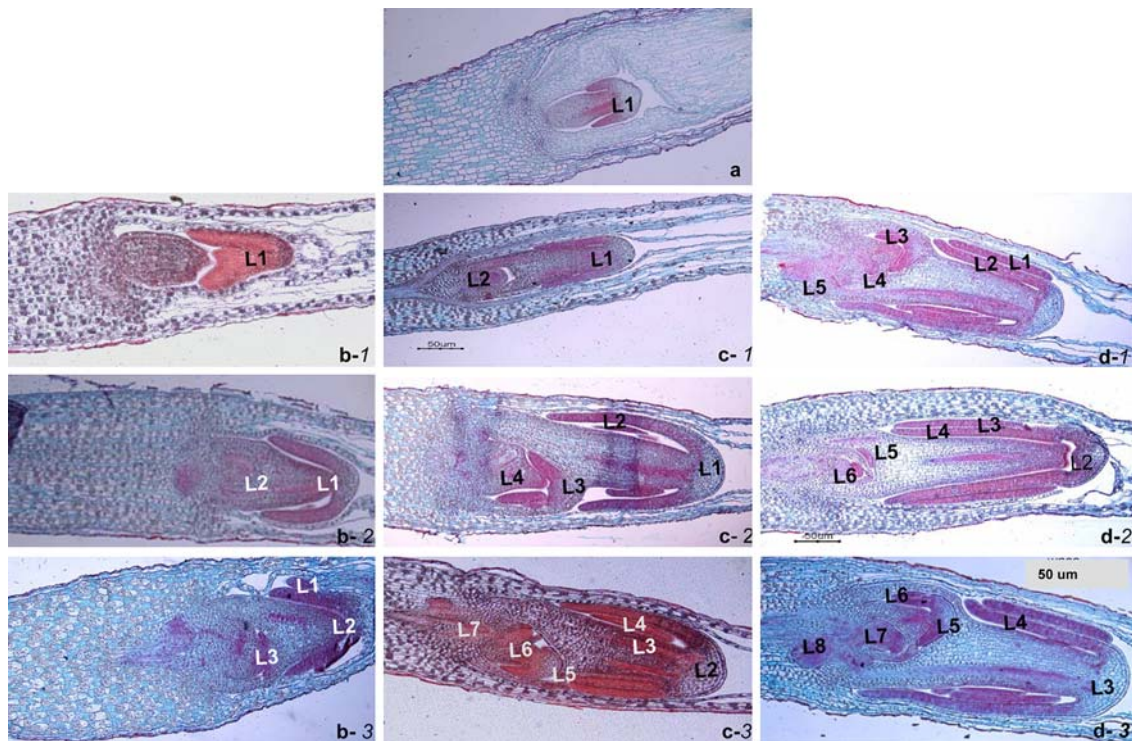


**Fig. 2** Effect of 4 and 25°C on seedlings of *P. hexandrum*. **a** Shriveling of cotyledonary leaves after 7 days at 4°C and senescence of aerial parts after 10 days. Functional leaf emerges 15 days after transfer of the seedlings to 25°C. **b** Senescence of aerial

parts (cotyledonary leaf and tubulate parts) as the underground parts (base of the tubulate cotyledons, hypocotyl and root system) remained healthy and alive 7–10 days after transfer of seedling from 25 to 4°C

up to 20 days but decreased with further exposure to 10°C. While  $\alpha$ -amylase activity (Fig. 4b), RS (Fig. 4c), nitrate (Fig. 4d), and NR activity (Fig. 4e) continued to increase with time, the first two showed a decline in Btc after 20 days. Starch (Fig. 4a), on the other hand, decreased in the first 10 days, only to increase steadily thereafter. In roots also, the decrease in starch (Fig. 4a) was coupled with an increase in  $\alpha$ -amylase (Fig. 4b) and RS (Fig. 4c) in the first 10 days. Although starch (Fig. 4a) increased steadily with time, the decline in the RS (Fig. 4c) was not as much. Surprisingly, the  $\alpha$ -amylase (Fig. 4b) activity also increased with time. The high contents of nitrate (Fig. 4d), which rose steadily up to 20 days, declined with further exposure to 10°C. On the other hand, the NR activity was low in the first 10 days, rose between 10 and 20 days and fell again with increasing time of exposure (Fig. 4e).

On transfer of the seedlings to 25°C 1 week after germination, the starch in the cot-leaf decreased about threefold in the first 10 days but increased steadily with time (Fig. 4a). A reverse trend was observed in the case of RS, where an initial increase in the first 10 days was followed by a very slight but gradual decrease with increasing time (Fig. 4c). The  $\alpha$ -amylase activity continued to increase in the first 20 days but declined with time (Fig. 4b). While NR activity continued to increase with time of exposure (Fig. 4e), the nitrate showed an increase up to 20 days, only to decline slightly with further exposure to 25°C (Fig. 4d). In the first 10 days of transfer, decrease in starch (Fig. 4a) in Btc was coupled with very high  $\alpha$ -amylase activity (Fig. 4b) and considerably high RS content (Fig. 4c). With increasing time, however, there was a slight decline in RS, and although not remarkable, there



**Fig. 3** Longitudinal sections of the node at the base of tubulate cotyledons of *P. hexandrum* seedlings stained with Safranin for 4 h and Fast green for a few seconds showing increasing number of functional leaf primordia (L1–8) formation with time at different

temperatures. **a** control at 0 day, **b 1–3**: 4°C, **c 1–3**: 10°C and **d 1–3**: 25°C where **a-1**, **b-1**, **c-1** represent 10 days, **a-2**, **b-2**, **c-2** represent 20 days and **a-3**, **b-3**, **c-3** represent 30 days of exposure, respectively

was a further increase in  $\alpha$ -amylase activity. The starch content also increased threefold (Fig. 4a). Between 20 and 30 days, a slight decline in starch (Fig. 4a) and  $\alpha$ -amylase activity (Fig. 4b) and a drastic decline in RS (Fig. 4c) were observed. While the nitrate increased several-fold in the first 10 days, further increase with time was not remarkable (Fig. 4d). On the other hand, the NR activity declined drastically in the first 10 days but increased slightly with time (Fig. 4e).

Very high  $\alpha$ -amylase activity did not correspond to either the absence of any change in starch content or concomitant increase in RS in the roots of seedlings transferred to 25°C (Fig. 4a–c). Although  $\alpha$ -amylase activity increased steadily with further exposure to this temperature, the RS content did not increase accordingly (Fig. 4b, c). Rather a decline in RS (Fig. 4c) was coupled with a slight increase in starch (Fig. 4a). High amounts of nitrate were coupled with persistently high NR activity (Fig. 4d, e). While NR activity remained steady, a slight decline in nitrate was observed with time (Fig. 4d, e).

#### Respiration and TDH

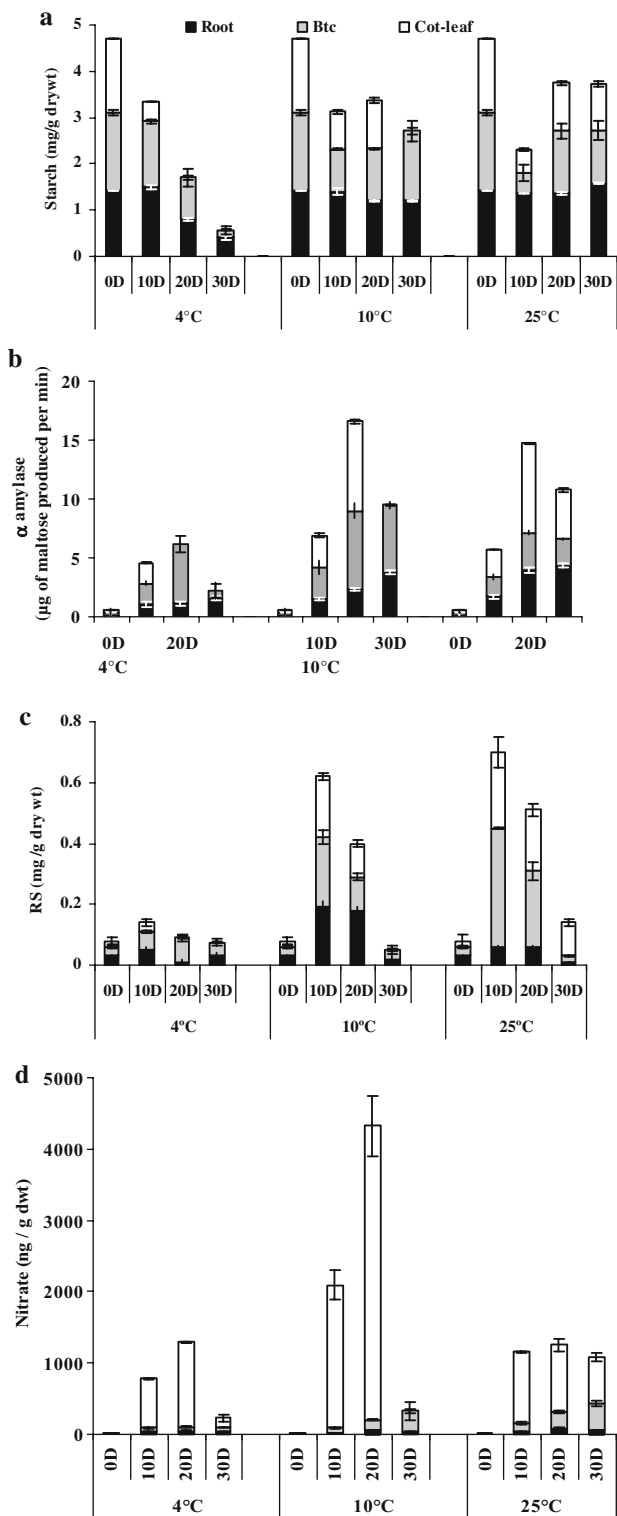
High rates of respiration and TDH activity were observed in the seedlings growing at 25°C, whereas a drastic

reduction in both respiration and TDH activity was observed in these at 4°C. Decline was also observed at 10°C in both respiration and TDH activity, although not as drastic as at 4°C (Fig. 4f, g).

#### Discussion

Temperature-dependent start and break of hypocotyl dormancy, the presence of pre-determined meristems and their consequent vegetative growth in the seedlings of *P. hexandrum* have been shown in the present study. The study also shows the senescence of aerial parts but survival of only belowground perennating organs at low temperatures (4°C). Under lab conditions, a higher temperature of 25°C was found to break the hypocotyl dormancy. Simply an exposure of the newly germinated seedlings to favorable temperature (25°C in the present study) was sufficient for the shriveling of cot-leaves and emergence and rapid growth of the functional leaves. Although *P. hexandrum* is a slow-growing plant that exhibits hypocotyl dormancy for prolonged periods even under ex situ conditions, the phenomenon of plastochrony was observed within 10–30 days at 25°C.

Functional leaf emergence within the year of germination and/or in the following season of growth has been



**Fig. 4** Changes in **a** starch, **b**  $\alpha$ -amylase activity, **c** reducing sugars, **d** nitrates, **e** nitrate reductase activity, **f** respiration and **g** total dehydrogenase activity in roots, base of tubulate cotyledonary leaves (*Btc*) and cotyledonary leaves (*cot-leaf*) in response to 0, 10, 20, and 30 days of exposure to 4, 10, and 25°C

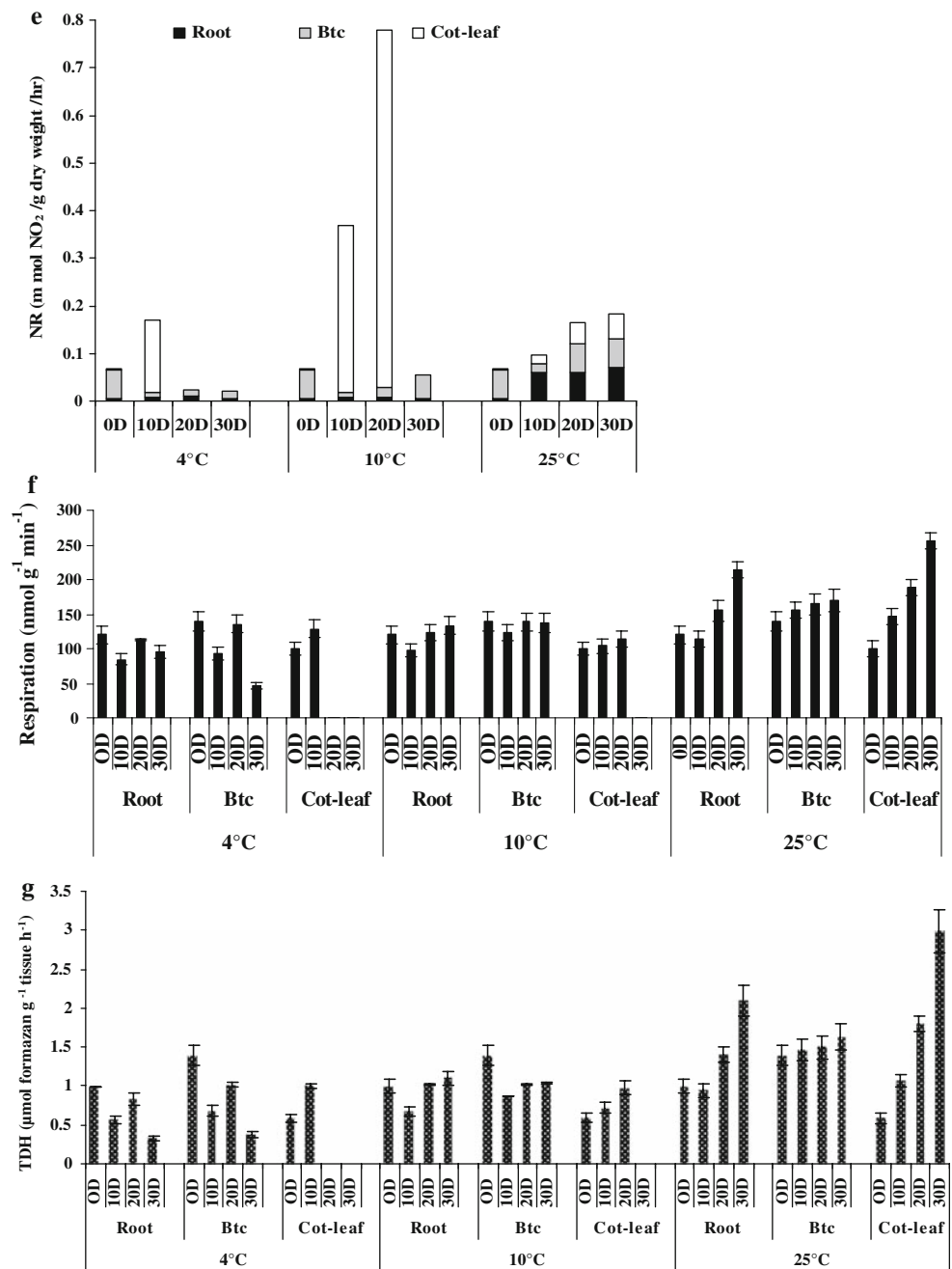
reported by Terabayashi (1987). Functional leaves probably emerged in their study whenever the temperature was favorable, and this was irrespective of the season of growth. In the natural habitats of *P. hexandrum*, the mean maximum temperature of the favorable season of growth is generally 25°C and the onset of winters is 10°C (i.e., the time when hypocotyl dormancy sets in and senescence of all aerial parts is initiated). Under lab conditions also, hypocotyl dormancy was initiated at 10°C as evident from the senescence of the aerial parts, quiescence or very slow development of the leaf primordia, and no functional leaf emergence (Fig. 3). When the seedlings were exposed to still lower temperatures, i.e., 4°C, all the aerial parts senesced and only the belowground parts remained healthy and alive. Interestingly, 4°C represents the root-zone temperature under a thick cover of winter snow under in situ conditions.

The phenotypic changes in growth that were observed in the present study in response to season and temperature were probably the adaptive responses of *P. hexandrum* seedlings. Being an alpine Himalayan plant with pronounced seasonality, it is natural that the seedlings of *P. hexandrum* would exhibit a high degree of adaptive specialization for avoiding the harsh alpine environment. This is generally achieved through a tight synchronization of growth processes with season (Körner and Spehn 2002). Several factors including photoperiods are important for this synchronization; the present study focused mainly on temperature. Of all the factors, temperature represents the most adverse conditions for *P. hexandrum*, i.e., the freezing winters. Lack of morphophysiological and anatomical adaptations for maximizing photosynthesis under light intensities of varying photoperiods as shown by Pandey et al. (2006) further indicated that light was not as limiting a factor as temperature.

Synchronization of growth as indicated by phenotypic responses to season and temperature were bound to involve major changes in carbon and nitrogen metabolism. Their proper investment, accumulation, and distribution to different seedling parts are necessary for desired changes in growth processes. Such changes in carbon and nitrogen metabolism are required for ensuring sufficient but easily available energy sources (Hoch et al. 2002) at each stage of growth. It is probably because of this that  $\alpha$ -amylase and RS (the easily available carbon source) increased, in all seedling parts, immediately after transfer to 4°C (Fig. 4b, c). This was coupled with a reduction in starch in cot-leaf and Btc during the first 10 days (Fig. 4a). On prolonged exposure (i.e., 20 days) to this unfavorable temperature (4°C), the large amounts of soluble sugars that accumulated in the Btc defined the growth of the predetermined



Fig. 4 continued



meristems prior to perennation and hence acclimation. Although there was no emergence of functional leaf at this temperature, ontogenic development of leaves was evident, albeit slow, from histological studies (Fig. 3b). Since the roots are the perennating organs, all the parameters related to sugars decreased in the seedling's parts on prolonged exposure to 4°C (Fig. 4a–c) with the exception of the roots, wherein an overall slight increase (1.36-fold) in  $\alpha$ -amylase was observed. On the whole, seedlings appeared to be preparing for an oncoming but prolonged period of low temperature. With further persistence of low temperature

(4°C) for 30 days, the *P. hexandrum* seedlings were left with no alternative but to enter into a phase of quiescence and perennation (personal observation).

An initial decline in growth and metabolism of the seedlings during the first 10 days at 10°C followed by slight increase after 20 days indicated a trend similar to that of 4°C. Since 10°C was less adverse than 4°C, the growth and ontogenic development of leaf primordia was also comparatively faster (Fig. 3c). The demand for the available form of carbohydrates at 10°C was also, therefore, more than that at 4°C (Fig. 4a). Thus, a sharper

decrease in starch in the Btc was coupled with a 6.2-fold increase in RS and an 11.86-fold increase in  $\alpha$ -amylase (Fig. 4a, b, c).

The comparatively fastest development of functional leaves and also plastochrony (Fig. 3d) was observed at 25°C, the optimum temperature for vegetative growth. The lowest content of starch in Btc—the site of leaf primordia (Fig. 4a–c)—coupled with an increase in  $\alpha$ -amylase (7.3-fold) and RS (10.54-fold) probably reflected the huge requirements for readily available carbon needed to meet the construction cost of the functional leaves.

Irrespective of temperature, maximum changes in nitrate and NR occurred when the readily available form of sugars (RS) was highest, especially during the initial 10 days after transfer. This appeared to indicate that the temperature-dependent availability of the carbon source, but not temperature itself, was an important regulator of nitrogen uptake and its reduction, as has been shown by Aslam and Oaks (1975) and Jackson et al. (1980). Despite the high correlation between nitrate and NR, an opposite trend was observed in the Btc of the seedlings growing at 4, 10, or 25°C during the first 10 days (Fig. 4d, e). The low NR, despite a steep increase in nitrate, was probably because the Btc is just a translocating organ where reduction of nitrate is not a major activity. The highest NR and nitrate levels in cot-leaf but low NR levels in roots can be explained by the fact that, while roots take up most of the nitrate from soil (Scheurwater et al. 2002), their reduction occurs in the photosynthetic organs (which is cot-leaf in the present study).

Loss of aerial parts of seedlings transferred from 25 to 4°C as compared to emergence of functional leaf and vegetative growth in the perennating belowground parts of seedlings transferred from 4 to 25°C further confirmed the temperature-dependent phenotypic changes. Quiescence in response to 4 and 10°C, and further development and growth of the seedlings at 25°C, indicated the preference of seedlings to remain quiescent without the aerial parts at the low temperatures that characterize the natural conditions of *P. hexandrum*. Investment of costly carbon and nitrogen assimilates in new structures like functional leaves before the oncoming harsh winters would be a wasteful process as these would invariably senesce at low temperatures. Rather, the maintenance of the predetermined meristems at Btc—a well-protected belowground part of the tubulate cotyledons—ensured the initiation of vegetative growth immediately with the advent of the short-growing season after winter dormancy. Predetermined meristems were probably maintained with the help of  $\alpha$ -amylase activity and increased accumulation of nitrate and starch at 4 and 10°C. With the return of favorable temperatures (25°C), the reserves stored in roots (starch and nitrate) were probably immediately utilized (evident from reduced starch and

increased RS in Btc coupled with an increase in respiration and TDH as compared to that at 4 or 10°C).

In conclusion, the present study showed that while maximum synthesis and utilization occurred at 25°C in cot-leaf and Btc for new structure (functional leaf) formation, maximum storage occurred at 4°C in roots.

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