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In vitro multiplication of *Quercus leucotrichophora* and *Q. glauca*: Important Himalayan oaks

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Abstract

Multiple shoots of *Quercus leucotrichophora* L. and *Q. glauca* Thunb. were induced from the intact embryos (decoated seeds) as well as from the cotyledonary nodes (with attached cotyledons but without radicle and primary shoot) of 3-weeks old *in vitro* grown seedlings on Woody Plant (WP; Lloyd and McCown, 1980) and Murashige and Skoog (MS; 1962) media supplemented with 6-benzyladenine (BA), either alone or in combination with gibberellic acid (GA_3)/ indole-3-butyric acid (IBA). BA (22.19 μM) was effective for induction of multiple shoots and addition of GA_3 to the medium further enhanced the shoot number and shoot height but resulted in shoot thinness. High frequency shoot multiplication was achieved using cotyledonary nodes. Shoots were further multiplied from the original explant on WP medium supplemented with BA (22.19 μM). Nearly 78% and 67% rooting was obtained in *Q. leucotrichophora* and *Q. glauca* microshoots (3–4 cm high), respectively on 1/2 strength WP medium supplemented with IBA (14.76 μM). However, this was associated with basal callus formation. Treatment with IBA (25–100 μM) for 24 or 48 h followed by transfer to PGR free 1/2 strength WP medium not only improved the rooting percentage but also avoided basal callus formation. IBA at 100 μM for 24 h was most effective (90% and 100% rooting in *Q. leucotrichophora* and *Q. glauca*, respectively). *In vitro* rooted plants were hardened and established in garden soil.

Growth performance of 6-month-old *in vitro* raised plants was compared with *ex vitro* plants (seedlings) of the same age. The photosynthesis and transpiration rates of eight months old *in vitro* and *ex vitro* raised plants of both species were measured under different light (0, 600, 900, 1200, 1500 and 2000 μmol $m^{-2}s^{-1}$) and temperature (20, 25, 30, 35 and 40 °C). Light optimum for photosynthesis was around 2000 μmol $m^{-2}s^{-1}$ in *Q. leucotrichophora* and around 1500 μmol $m^{-2}s^{-1}$ in *Q. glauca* whereas optimum temperature for photosynthesis was 25 °C in *Q. leucotrichophora* and 30 °C in *Q. glauca*. The rate of transpiration at different temperatures (20–40 °C), in the two species, increased with increase in the light intensity up to the highest level, i.e., 2000 μmol $m^{-2}s^{-1}$. Temperatures beyond 35 °C adversely affected the rate of transpiration in *in vitro* raised as well as *ex vitro* plants of both the species. *In vitro* raised and hardened plants of both the species were comparable to *ex vitro* plants in terms of gas and water vapour exchange characteristics, within the limits of this study.

Abbreviations: BA – 6-benzyladenine; GA_3 – gibberellic acid; IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962) medium; PAR – photosynthetically active radiation; PGR – plant growth regulator; PPFD – photosynthetic photon flux density; WP – Woody Plant (Lloyd and McCown, 1980) medium

Introduction

Oak (*Quercus* species), a large genus of trees and shrubs, either deciduous or evergreen is represented by 5 evergreen species, namely *Q. glauca* (phaniyat oak), *Q. leucotrichophora* (banj oak), *Q. floribunda* (tilonj oak), *Q. lanuginosa* (rianj oak) and *Q. semecarpifolia* (brown oak) in the Central Himalaya between 1000

and 3600 m amsl (Champion and Seth, 1968). These species play a vital role not only in soil and water conservation but also contribute significantly to the sustenance of rural ecosystems. The wood is used as fuel and for making agricultural tools, leaves as green fodder and leaf litter as cattle bedding. Therefore, they have been exploited indiscriminately. Natural regeneration through seed is poor (Troup, 1921). Irregular fructification, consumption of seeds by animals (Troup, 1921) and loss of viability following storage for extended periods (Chalupa, 1995) further exacerbates the problem of regeneration. Clonal (vegetative) propagation of oaks through stem cuttings, in general, is only marginally effective when cuttings from young trees are used while cuttings collected from mature (and elite) trees are difficult to propagate (Bhardwaj et al., 1996). Tamta et al. (2000) have, however, reported some success in rooting of stem cuttings taken from mature trees of *Q. leucotrichophora*.

Tissue culture could be useful in overcoming above cited difficulties (Bisht et al., 1998). So far micropropagation of the above mentioned five oak species has not been reported. Although *in vitro* propagation via axillary shoot multiplication has been reported for some other oak species, namely *Q. robur* and *Q. petraea* (Vieitez et al., 1985; Favre and Juncker, 1987; Chalupa, 1988; San-Jose et al., 1990), *Q. suber* (Pardos, 1981; Bellarosa, 1989; Manzanera and Pardos, 1990), *Q. shumardii* (Bennet and Davies, 1986), *Q. acutissima* (Sato et al., 1987), *Q. serrata* (Ide and Yamamoto, 1987) and *Q. rubra* (Schwarz and Schlarbaum, 1993), micropropagation using cotyledonary nodes of *in vitro* raised seedlings has not been reported for any of the oak species. Many species of *Quercus* have a great stump sprouting ability, due to the presence of several dormant buds that are preformed at a very early stage of development of the tree. Successful initiation of cultures and regeneration in some oak species has been carried out using basal shoots or stump sprouts of mature trees (Vieitez et al., 1985; San-Jose et al., 1988; Chalupa, 1988; Vieitez et al., 1994). Somatic embryogenesis has also been reported in several oak species (Gingas, 1991; Chalupa, 1995; Endemann and Wilhelm, 1999; Wilhelm, 2000 and references therein).

Following micropropagation, the most important step for field transfer is transition during hardening from *in vitro* to an *ex vitro* environment; this also affects subsequent field performance. If not properly hardened survival of *in vitro* raised plants under *ex vitro* conditions is poor mainly due to improper development of cuticular waxes, non-functional stomata, water loss due to excessive transpiration, poor root system and susceptibility to pathogens (Ziv, 1995; Bisht et al., 1998). However, in the course of hardening, the micropropagated plants gradually overcome these inadequacies and adapt to *ex vitro* conditions.

Plant survival, growth and productivity are intimately coupled with the aerial environment through processes such as energy exchange, loss of water vapour in transpiration and uptake of carbon dioxide in photosynthesis (Jarvis et al., 1988; Stoutjesdijk and Barkman, 1992). The water vapour exchange rate affects the energy budget and transpiration of leaves and consequently the physiology of the whole plant (Gates, 1975; Chandra and Dhyani, 1997). Therefore, data on physiological parameters such as gas and water vapour exchange are likely to provide valuable information regarding the suitability of tissue culture raised plants for field plantations.

In view of the importance of oaks and the problems associated with their natural regeneration, two species, namely *Q. leucotrichophora* L. and *Q. glauca* Thunb. have been selected, for the present study with the following two objectives: First objective: to develop an efficient *in vitro* micropropagation methodology through multiple shoot formation (and subsequent rooting) from seed as well as from cotyledonary nodes (1–2 cm, with attached cotyledons). Second objective: to evaluate the performance of *in vitro* raised and hardened plants on the basis of selected morphological and physiological parameters in comparison to those of *ex vitro* plants of the same age. The effect of different light intensities and temperatures on photosynthesis and water vapour exchange characteristics have also been examined.

Material and methods

Plant material

Seeds of *Quercus leucotrichophora* L. and *Q. glauca* Thunb. were collected from trees growing in the forests at Katarmal (1250 m amsl) and Ranman (1500 m amsl), Dist. Almora, Uttaranchal, India, respectively. The seeds were separated from the cupule, wetted in 100 ml of detergent solution (labolene, 0.1%, v/v; 10 min), and then washed under running tap water for 5 min. Subsequently the seeds were rinsed in distilled water (×4), sequentially treated in solutions containing a systemic fungicide (bavistin, 0.2%, w/v;

30 min), and an antioxidant (ascorbic acid, 0.02%, w/v; 30 min). Finally they were surface disinfected with an aqueous solution of mercuric chloride (0.05%, w/v; 10 min). Each treatment was followed by repeated washings (×4) with sterile distilled water under aseptic conditions.

Excision and culture medium

After removing the seed coat from disinfected seeds, the decoated seeds (embryos) were inoculated on water agar (0.8%, w/v) medium containing sucrose (3.0%, w/v). The germinating embryos, either intact or after removing the radicle and primary shoot from the seedlings (cotyledonary nodes), were transferred to Murashige and Skoog (MS; 1962) or Woody Plant (WP; Lloyd and McCown, 1980) medium containing sucrose (3%, w/v) and agar (0.8%, w/v) and supplemented with various concentrations of plant growth regulators (PGRs): 6-benzyladenine (BA; 2.22–22.19 μM), gibberellic acid (GA_3; 2.89 μM) and indole-3-butyric acid (IBA; 1.89 μM). The pH of the medium was adjusted to 5.8, and the medium was poured into 250 ml Erlenmeyer flasks (100 ml medium per flask) and autoclaved (1.05 kg cm^{-2}, 121 °C, 20 min). Each treatment consisted of 24 explants (3 explants per flask) and all experiments were repeated at least twice. The cultures were maintained at 25 ± 1 °C in a 16 h light and 8 h dark cycle, with irradiance (42 μmol $m^{-2}s^{-1}$) by cool fluorescent tubes (Philips; 40 W). Subculturing was carried out at 5–6 weeks intervals and data on shoot number and shoot length were recorded 30 days after subculture.

In vitro shoot formation

When the initial explants (i.e. intact embryos) were inoculated on MS or WP media, supplemented with different combinations of PGRs, more than one shoot emerged (termed as the 'first crop' of shoots) in some PGR combinations (Table 1; Figure 1B) whereas when the cotyledonary nodes (following removal of radicle and the primary shoot), were cultured on the same media, the number of shoots formed was found to increase (Table 2; Figure 1C). After harvesting the first crop of microshoots, both the intact embryos and the cotyledonary nodes were subcultured for additional shoot formation on fresh medium (WP with 22.19 μM BA); about 2–3 shoots per intact embryo and 7–11 shoots per cotyledonary node were obtained, these are referred to as the 'second crop' of microshoots. This could be continued up to the second subculture in case of intact embryos and up to the fourth subculture in case of cotyledonary nodes; after this, the cotyledons in the intact embryos as well as the cotyledonary node experiments appeared highly desiccated.

Rooting of shoots and transfer of plantlets to soil

For root induction, microshoots (2.0–3.0 cm height with 2–3 leaflets) were transferred to 1/2 strength WP medium containing sucrose (3.0%; w/v), phytagel (0.25%; w/v) and indole-3-butyric acid (IBA; 0.44–24.61 μM). Excised microshoots (n = 24) were also treated with IBA (25–100 μM) for 24 or 48 h only and then transferred to PGR free 1/2 strength WP medium. After 15 days, the shoots with well developed roots were taken out of the culture medium, the roots gently washed with water to remove traces of phytagel and the plantlets were then transferred to small plastic cups (6.1 cm diameter, 8.5 cm height) containing non-sterile soil and farmyard manure (3:1, v/v); these were kept in a mist chamber (25 °C, 80% RH) for acclimatization. One-month-old acclimatized plantlets were transferred to polybags containing the same potting mixture and placed under outdoor conditions with partial shade in the beginning and then moved to a place where the seedlings received full sunlight. Six months after transfer to soil, the growth performance of plantlets raised via the first crop shoots was recorded and later on compared with that of plantlets derived from the second crop shoots as well as with that of *ex vitro* seedlings of the same age (age of *ex vitro* plants was taken from the day of seedling emergence).

Gas and water vapour exchange

Eight months after transfer to soil, four sets of plants (*in vitro* raised plants from the second crop of shoots and *ex vitro* plants of the same age for the corresponding two species; four plants for each set) were used for carbon assimilation and water vapour exchange studies. Measurements were carried out on four upper undamaged, fully expanded and healthy leaves of each plant with the help of a closed portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, Nebraska, USA). To study the effect of light on gas and water vapour exchange, leaves were exposed to different photosynthetic photon flux densities (PPFD) viz., 100, 600, 900, 1200, 1500 and 2000 μmol m^{-2} s^{-1} with the help of an artificial light source (Model LI- 6400-02; light emitting silicon diode; LI-COR), fixed on the top of the leaf chamber. Photosynthetically active radiation (PAR) was recorded with the help of a

Table 1. Effect of media composition and plant growth regulators (μM) on multiple shoot formation in intact embryos of *Q. leucotrichophora* and *Q. glauca*

Treatments			*Q. leucotrichophora*				*Q. glauca*			
[a]PGRs			MS		WP		MS		WP	
BA	GA_3	IBA	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE
0.0	0.0	0.0	1.0±0.05	3.2±0.47	1.0±0.09	4.4±1.05	0.8±0.09	4.5±0.73	1.2±0.73	3.6±0.79
2.22	0.0	0.0	1.3±0.12	5.5±0.61	2.3±0.36	3.9±0.30	1.1±0.15	5.2±0.83	1.5±0.45	4.3±0.83
4.44	0.0	0.0	1.5±0.41	5.6±0.61	3.4±0.73	3.2±0.37	2.0±0.26	3.0±0.30	4.5±0.71	2.0±0.48
22.19	0.0	0.0	3.5±0.71	3.3±0.27	5.0±1.41	2.4±0.26	3.0±0.62	1.7±0.23	5.1±1.47	1.3±0.05
2.22	2.89	0.0	2.8±0.44	2.7±0.42	2.4±0.54	2.7±0.28	2.3±0.40	3.4±0.61	5.0±0.50	2.3±0.19
4.44	2.89	0.0	2.3±0.20	3.3±0.93	3.8±0.17	3.2±0.25	3.5±0.67	3.1±0.09	4.0±0.71	3.5±0.38
22.19	2.89	0.0	3.8±0.13	3.7±0.06	5.1±0.75	3.5±0.51	4.1±0.48	3.9±0.19	6.2±0.54	4.8±0.79
2.22	0.0	4.92	1.8±0.59	1.8±0.17	1.5±0.41	0.7±0.12	1.0±0.09	2.6±0.60	1.3±0.12	2.0±0.31
4.44	0.0	4.92	2.0±0.24	1.8±0.18	2.2±0.17	1.2±0.27	1.9±0.29	1.7±0.31	1.9±0.62	1.9±0.32
22.19	0.0	4.92	0.9±0.14	3.2±0.94	1.8±0.62	1.1±0.09	0.5±0.11	4.6±0.72	2.0±0.24	3.0±0.22
LSD ($p = 0.05$)			1.57	2.11	2.32	1.59	1.36	1.90	2.38	1.84

ANOVA SUMMARY TABLE

Source	*Q. leucotrichophora*						*Q. glauca*					
	Shoot No.			Avg. shoot length			Shoot No.			Avg. shoot length		
	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio
PGR concentration	9	8.00	10.64*	9	24.59	8.68*	9	13.32	15.17*	9	11.27	7.52*
Media type	1	8.51	11.31*	1	0.00	0.00ns	1	22.69	25.83*	1	60.72	40.52*
PGR con. × Media Type	9	1.04	1 .38ns	9	1.61	0.57ns	9	1.58	1.803ns	9	1.37	0.92ns
Error	38	0.75		38	2.83		38	0.87		38	1.50	

*Level of significance at 0.01; ns: not significant.
[a]PGRs: Plant growth regulators.
MS: Murashige and Skoog (1962) medium, WP: Woody Plant (Lloyd and McCown, 1980) medium, SE: Standard error
Data were recorded after 30 days; all values are an average of 24 explants and the experiment was repeated twice with qualitatively similar results.

Table 2. Effect of media composition and plant growth regulators (μM) on multiple shoot formation in cotyledonary nodes of *Q. leucotrichophora* and *Q. glauca*

Treatments			*Q. leucotrichophora*				*Q. glauca*			
[a]PGRs			MS		WP		MS		WP	
BA	GA_3	IBA	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE	No. of shoots/seed ±SE	Avg. shoot length (cm) ±SE
0.0	0.0	0.0	0.97±0.03	5.0 ±0.07	0.8±0.07	6.2±0.12	0.97±0.07	3.6±0.24	1.4±0.47	4.0±0.64
2.22	0.0	0.0	2.9±0.05	5.8±0.23	3.6±0.07	5.0±0.09	2.7±0.09	4.9±0.07	3.0±0.40	5.3±0.24
4.44	0.0	0.0	3.8±0.12	6.7±0.2	4.7±0.10	3.4±0.07	3.1±0.10	4.1±0.31	4.1±0.29	3.9±0.02
22.19	0.0	0.0	4.6±0.14	5.7±0.22	12.9±0.95	4.2±0.08	6.1±0.07	1.7±0.11	7.4±0.15	3.2±0.16
2.22	2.89	0.0	1.9±0.05	4.7±0.10	6.4±1.18	3.3±0.12	1.0±0.14	1.8±0.05	1.3±0.30	1.8±0.18
4.44	2.89	0.0	5.2±0.28	8.2±0.04	8.3±0.98	8.8±0.12	6.5±0.12	4.7±0.13	8.5±0.83	4.8±0.15
22.19	2.89	0.0	6.1±0.07	6.5±0.23	13.3±0.72	4.6±0.18	8.8±0.13	4.1±0.69	9.3±1.23	3.8±0.04
2.22	0.0	4.92	1.7±0.10	6.1±0.22	1.0±0.09	4.5±0.45	1.4±0.13	3.6±0.10	1.9±0.19	4.1±0.22
4.44	0.0	4.92	2.8±0.13	5.0±0.09	1.0±0.29	4.1±0.18	3.0±0.12	3.2±0.14	4.2±0.59	2.5±0.12
22.19	0.0	4.92	0.97±0.07	5.0±0.49	0.9±0.10	4.5±0.62	0.8±0.07	2.0±0.05	1.5±0.18	2.3±0.07
LSD ($p = 0.05$)			0.44	0.42	0.81	0.61	0.96	0.40	0.54	0.46

ANOVA SUMMARY TABLE

Source	*Q. leucotrichophora*						*Q. glauca*					
	Shoot No.			Avg. shoot length			Shoot No.			Avg. shoot length		
	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio	DF	MS	*F*-Ratio
PGR concentration	9	62.97	64.00*	9	23.67	90.07*	9	50.47	62.75*	9	6.65	19.43*
Media type	1	73.92	75.13*	1	16.87	64.21*	1	9.36	11.63*	1	1.29	3.78**
PGR con. × Media Type	9	18.18	18.48*	9	5.52	2.09ns	9	0.51	0.64ns	9	0.56	1.63ns
Error	38	0.98		38	0.26		38	0.80		38	0.34	

*Level of significance at 0.01; **level of significance at 0.05; ns: not significant.
[a]PGRs: Plant growth regulators.
MS: Murashige and Skoog (1962) medium, WP: Woody Plant (Lloyd and McCown, 1980) medium, SE: Standard error. Data were recorded after 30 days; all values are an average of 24 explants and the experiment was repeated twice with qualitatively similar results.

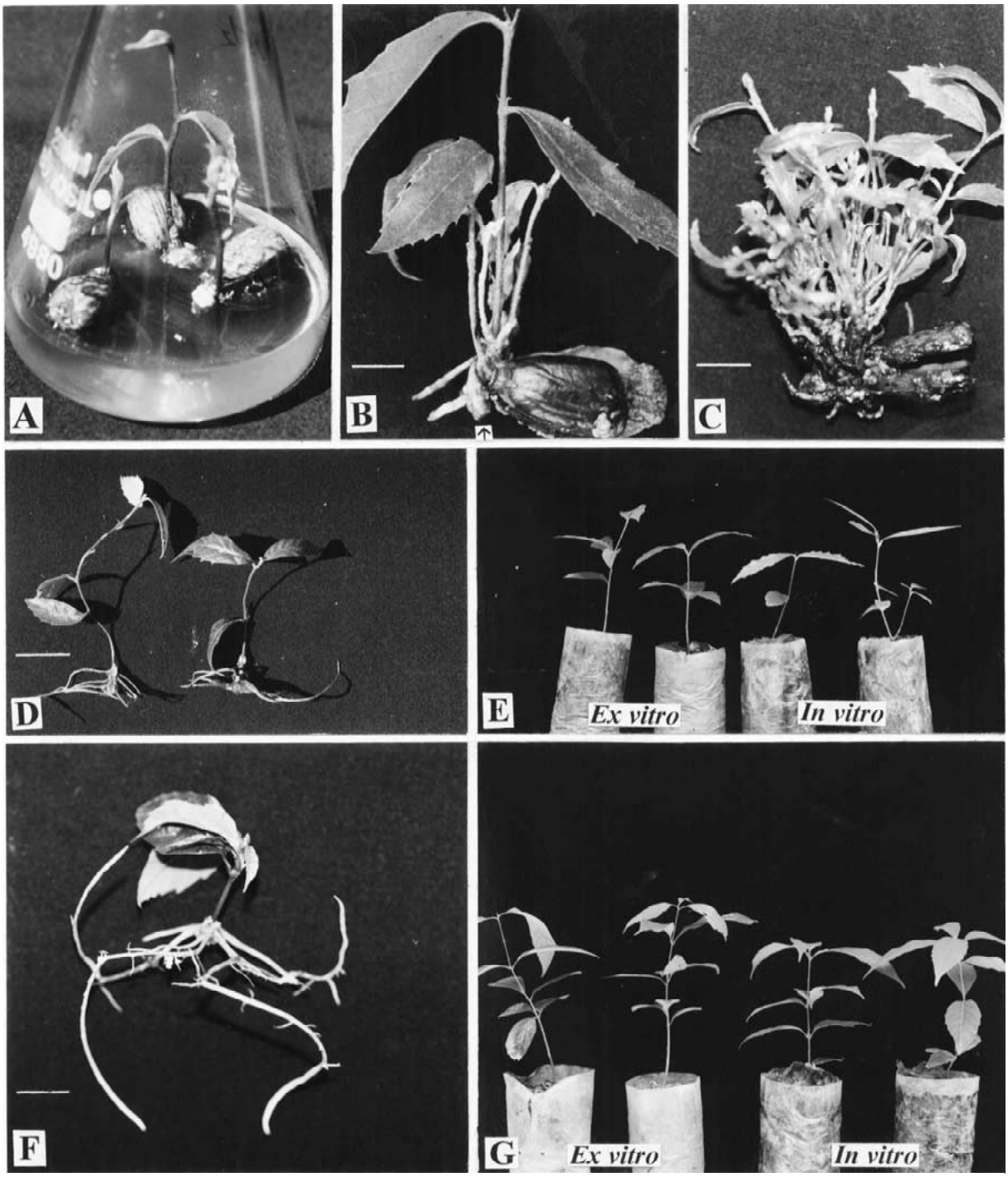

Figure 1. *(A)* Germinating seeds of *Q. leucotrichophora* in water agar medium; *(B)* multiple shoot formation from intact embryo (the arrow indicates position of a root that was excised before photography; bar=1.08 cm), and *(C)* from cotyledonary nodes (bar=1.33 cm) of *Q. leucotrichophora*; *(D)* rooting of microshoots of *Q. leucotrichophora* (bar=1.75 cm); *(E) ex vitro* and *in vitro* raised plants of *Q. leucotrichophora* after six months of transfer to soil; *(F)* rooting of microshoots of *Q. glauca* (bar=1.09 cm); *(G) ex vitro* and *in vitro* raised plants of *Q. glauca* after six months of transfer to soil.

quantum sensor kept in the range of 660–675 nm wave radiation, fixed at the leaf level. The rate of dark respiration was measured by maintaining the leaf in the cuvette at zero irradiance. To avoid any radiation from the outside, the leaf chamber was covered with black cloth throughout the respiratory measurements. Air flow rate (500 μmol s^{-1}), CO_2 concentration inside the leaf chamber (350 $\pm$ 5 ppm) and relative humidity (55 $\pm$ 5%) were kept nearly constant throughout the experiment. Since steady state photosynthesis is reached within 30–45 min, the leaves were kept for about 45–60 min under each set of light conditions before the observations were recorded. All measurements were carried out at different temperatures (20, 25, 30, 35 and 40 °C).

Statistical analyses

Least significant difference was calculated following the method of Snedecor and Cochran (1967). The effects of different concentrations of PGRs and media type were quantified and the level of significance was determined by analysis of variance (Wilkinson, 1986). Analysis of variance, ANOVA (SYSTAT of SPSS Inc., Chicago, USA) were used to test the differences in gas and water vapour exchange characteristics under the interactive effect of different light intensities and temperatures of *ex vitro* and *in vitro* raised plants. Rate of photosynthesis and transpiration was used as a dependent variable of light intensities and temperatures.

Results and discussion

Establishment of shoot cultures

Seeds of *Q. leucotrichophora* and *Q. glauca* germinated readily and the emerging seedlings became greenish within 7 days of inoculation and almost 100% germination was achieved in 3 weeks in water agar medium (Figure 1A). Culture of intact germinating embryos and excised cotyledonary nodes (size: approx. 1.0 cm, with two intact, attached cotyledons) from germinated embryos on MS or WP media, supplemented with various concentrations of BA, GA_3 and IBA resulted in shoot formation; however, the number of shoots formed varied with the treatment (Tables 1 and 2). Each intact embryo or cotyledonary node when cultured on PGR free media, developed into a complete plantlet with profuse root system. The frequency of shoots formed per intact embryo increased with increasing concentration of BA in the medium in both the species. The highest concentration (22.19 μM) of BA when used alone, was found to be quite effective in *Q. leucotrichophora* and in *Q. glauca* for shoot induction and WP medium gave better response compared to MS medium (Table 1 and Figure 1 B). The results of ANOVA show that PGR concentration and media type significantly ($p = 0.01$) improved shoot number as well shoot length of both the species, except for shoot length in *Q. leucotrichophora* (Table 1). However, the interaction of PGR concentration and media type was non significant (Table 1).

It was observed that addition of GA_3 (2.89 μM) to the medium containing 22.19 μM BA further enhanced the number of shoots formed as well as average shoot height. This combination of BA and GA_3 gave good response both in *Q. leucotrichophora* and in *Q. glauca* but the results were not significantly ($p = 0.05$) different in comparison to when BA was used alone. When cotyledonary nodes (size 1.0 cm, with two intact, attached cotyledons) excised from the germinating embryos were cultured on media with the same composition, a trend similar to that observed above was seen but with a higher number of shoots formed (Figure 1C). A smaller number of shoots were formed when intact embryos were used, which may be due to apical dominance. A similar pattern was reported by Bressan et al. (1982) in rose and by Hutchinson (1984) in apple where the nodal explants produced more shoots than the apical explants. BA at 22.19 μM induced multiple shoot buds in all explants within a week. The buds appeared as small green protuberances on the cotyledonary nodes that elongated into leafy shoots. The maximum number of shoots per explant was obtained on media supplemented with BA (22.19 μM) and GA_3 (2.89 μM) (Table 2), as was also the case in experiments with intact embryos (Table 1). Tables 1 and 2 indicate that the frequency of shoot formation was higher on WP medium than on MS medium. The results of ANOVA show that the interaction of growth regulator concentration and media type significantly ($p = 0.01$) improved the shoot number in *Q. leucotrichophora* (Table 2). Although the number of shoots formed per embryo or cotyledonary node was higher on medium supplemented with 22.19 μM BA and 2.89 μM GA_3, the shoots were thin and long (Table 2) compared to shoots obtained at other PGR combinations where the shoots had normal dark green leaves with thick stems. BA induced axillary shoot proliferation from the cotyledonary nodes of seedlings

has also been reported in other tree species, e.g., *Alnus glutinosa* (L.) Gaertn (Perinet and Lalonde, 1983). The addition of IBA to BA containing medium resulted in a reduced frequency of shoots (Tables 1 and 2).

The WP medium supplemented with 22.19 μM BA gave the second best response in terms of number of shoots formed as well as good shoot height (Table 2) without causing thinness of the shoots that was observed on a medium containing BA (22.19 μM) + GA_3 (2.89 μM). Therefore, the shoot cultures were multiplied by subculturing the embryos (without root) and the cotyledonary nodes (with cotyledons) on medium containing only 22.19 μM BA. The newly formed shoots were harvested and the explants were further subcultured with a decrease in the number of shoots formed and in their average height with each subculture (Table 3). About 7–11 shoots per cotyledonary node could be obtained after the first subculture; this value is several times higher than for shoots obtained from intact embryos (2–3 shoots per intact embryo) and that reported for other *Quercus* species, for example, only 2–3 shoots developed per nodal explant in *Q. robur* (Chalupa, 1988). BA was found to be an effective cytokinin as it stimulated higher shoot formation in comparison to other cytokinins tested in *Q. robur* (Chalupa, 1984, 1988). BA also significantly promoted *in vitro* growth and proliferation of shoots in *Q. robur* (Vieitez et al., 1985; Favre and Juncker, 1987) and was effective for shoot multiplication of *Q. shumardii* (Bennett and Davies, 1986). BA, its riboside and nucleotides are naturally occurring cytokinins in plant tissues (Nandi et al., 1989) and are relatively stable in comparison to other cytokinins (Letham and Palni, 1983); this may explain the improved response obtained with BA.

Induction of rooting in microshoots

The effect of different concentrations of IBA (0.44–24.61 μM) on *in vitro* rooting of microshoots (avg. height 2.0–3.0 cm with 2–3 leaflets) of *Q. leucotrichophora* and *Q. glauca* is summarized in Table 4. Half strength, PGR-free WP medium failed to induce roots in microshoots even after 30 days of culture but supplementing the medium with 14.76 μM IBA resulted in rooting (77.8% and 66.7% in *Q. leucotrichophora* and *Q. glauca* microshoots, respectively; Table 4). These treatments, however, resulted in some basal callus formation, which is not desirable as it adversely affects the survival of plantlets in the field. Therefore, the excised microshoots were also treated with higher concentrations of IBA (25–100 μM) for 24 or 48 h only and then placed in PGR free 1/2 strength WP medium. Treatment with 100 μM IBA for 24 h not only favoured early rooting by 15 days but also improved the rooting success (90% in *Q. leucotrichophora* and 100% in *Q. glauca*) without the basal callus formation (Table 4 and Figure 1D, F). The average number of roots per shoot (4.6 in *Q. leucotrichophora* and 3.7 in *Q. glauca*) and the average length of the longest root (3.6 cm and 3.8 cm in *Q. leucotrichophora* and *Q. glauca*, respectively) also was higher than when exposure to continuous IBA was used. This method of rooting was also found suitable in *Q. suber* (Manzanera and Pardos, 1990).

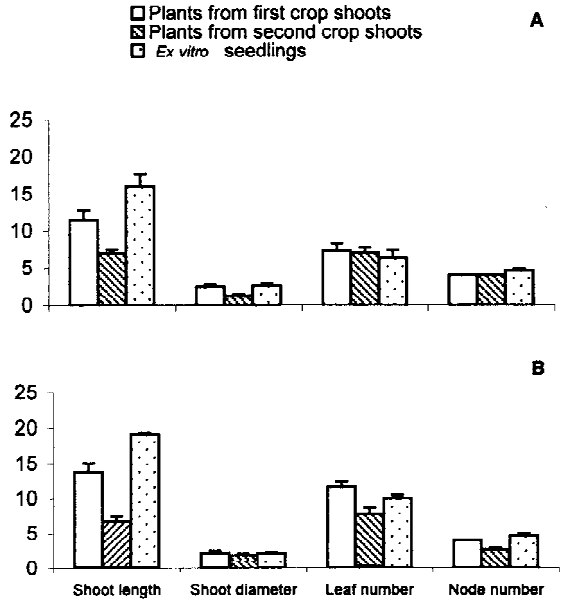

Figure 2. Comparison of *in vitro* raised plants with *ex vitro* seedlings. *(A) Q. leucotrichophora*; *(B) Q. glauca*. Values are an average of three seedlings. Shoot diameter (mm; $\times 10$) was recorded 2 cm from the ground level; shoot length is in cm. Data were recorded six months after transfer of *in vitro* raised plants to soil and the seedling age was also six months (following seedling emergence). Data for plants from the second crop shoots and *ex vitro* seedlings were recorded at the same time while data for plants from the first crop shoots were recorded at an earlier date (bars indicate $\pm$ SE).

Acclimatization and field establishment

The well rooted plantlets were acclimatized with 95% survival; these grew well and appeared healthy. The plantlets raised from the first crop of shoots grew more vigorously than plantlets derived from the second crop of shoots, but the differences were not statistically sig-

Table 3. Effect of repeated subculturing on initial explants using intact embryos and cotyledonary nodes in WP medium

Explant type	Subculture	*Q. leucotrichophora*		*Q. glauca*	
		No. of shoots/explant ±SE	Length of longest shoot (cm)±SE	No. of shoots/explant ±SE	Length of longest shoot (cm)±SE
Intact embryo	Initial	5.0±1.41	4.9±1.12	5.1±1.47	2.9±0.39
	After 1st subculture	2.3±0.72	2.8±0.59	2.8±0.95	1.9±0.05
	After 2nd subculture	1.4±0.25	1.2±0.36	1.3±0.27	1.4±0.24
Cotyledonary node	Initial	12.9±0.95	6.0±0.07	7.4±0.15	4.5±0.21
	After 1st subculture	10.7±1.19	5.3±0.39	7.0±0.24	3.4±0.62
	After 2nd subculture	8.3±0.98	4.2±0.49	4.7±0.72	3.2±0.36
	After 3rd subculture	5.3±0.72	4.0±0.47	3.7±0.54	2.9±0.05
	After 4th subculture	4.0±0.47	3.1±0.47	3.0±0.47	2.1±0.26

The medium was supplemented with BA (22.19 μM), SE: Standard error. All values are an average of 24 explants and the experiment was repeated once with qualitatively similar results.

Table 4. Effect of continuous exposure or short treatments with IBA on *in vitro* rooting of *Q. leucotrichophora* and *Q. glauca* microshoots

Treatment	*Q. leucotrichophora*			*Q. glauca*		
[a]**Continuous exposure to IBA (μM)**	% Rooting	Avg. no. of roots/shoot ±SE	Length of longest root (cm)±SE	% Rooting	Avg. no. of roots/ shoot±SE	Length of longest root (cm)±SE
0.0	0	0	0	0	0	0
0.44	0	0	0	0	0	0
2.46	0	0	0	11.1	0.7±0.45	0.5±0.41
4.92	22.2	1.3±0.72	1.3±0.54	22.2	1.7±0.72	1.2±0.59
7.38	33.3	1.8±0.95	1.7±0.72	22.2	1.7±0.72	1.5±0.62
9.84	33.3	0.8±0.63	0.7±0.56	33.3	1.8±0.76	1.6±0.71
14.76	77.8	2.6±0.32	2.9±0.31	66.7	2.8±0.33	2.7±0.31
24.61	33.3	1.5±0.85	2.1±0.85	33.3	0.8±0.63	0.6±0.48
LSD (p=0.05)		2.12	1.82		2.01	1.72
[b]**Short treatment with IBA (μM) for 24 or 48 h**						
0 (24)	0	0	0	0	0	0
(48)	0	0	0	0	0	0
25 (24)	44.4	1.5±0.41	4.3±0.32	0	0	0
(48)	33.3	2.5±1.18	3.3±1.33	0	0	0
50 (24)	33.3	1.5±0.85	1.7±0.76	22.2	4.2±0.83	2.8±0.12
(48)	44.4	3.3±0.72	3.0±0.41	55.6	1.8±0.83	2.4±1.01
75 (24)	55.6	3.5±0.24	3.9±1.00	55.6	1.3±1.09	1.1±0.90
(48)	33.3	2.0±0.47	3.2±0.36	33.3	2.8±0.51	3.9±0.41
100 (24)	90.0	4.6±0.43	3.6±0.26	100.0	3.7±0.27	3.8±0.35
(48)	77.8	4.0±0.82	3.4±0.66	66.7	3.5±0.24	3.9±0.71
LSD (p=0.05)		2.27	2.34		1.96	1.86

SE: Standard error

[a]Data were recorded 30 days after transfer to medium. After short treatment to IBA (24 or 48 h) the microshoots were transferred to 1/2 strength PGR-free medium. [b]Data were recorded 15 days after transfer to medium; each treatment consisted of 8 flasks with 3 microshoots per flask, n = 24.

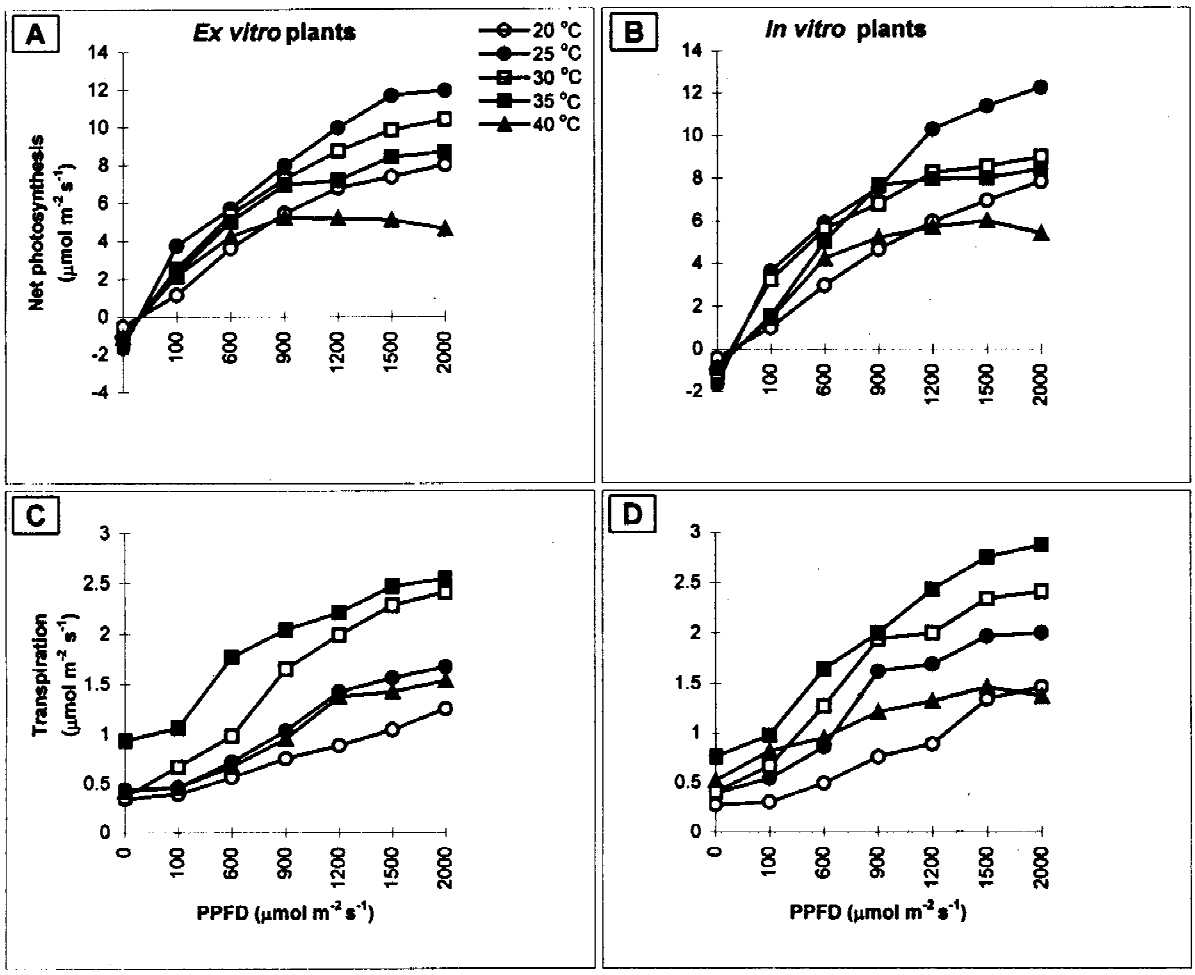

Figure 3. Comparison of gas and water vapour exchange rates in *ex vitro* (*A* and *C*; seedlings age: 8 months) and *in vitro* raised (*B* and *D*; 8 months following transfer to soil) plants of *Q. leucotrichophora* at varying photosynthetic photon flux density (PPFD; 0, 100, 600, 900, 1200, 1500 and 2000 μmol m^{-2} s^{-1}) and temperature (20, 25, 30, 35 and 40 °C). Symbols for different temperatures in Figures *B, C* and *D* are as in Figure *A*.

nificant. The *ex vitro* plants (seedlings) of the same age were taller than the *in vitro* raised plantlets, differences in the shoot diameter, number of leaves, number of nodes and number of internodes were, however, minor (Figure 2; also see Figure 1E, G).

The effect of different temperatures and light intensities on the rates of photosynthesis and transpiration of *ex vitro* and *in vitro* raised plants from the second crop shoots of *Q. leucotrichophora* are shown in Figure 3. Plants from the second crop shoots were used for comparison since these plants were of comparable age with *ex vitro* raised plants. Photosynthesis increased with increasing light intensity up to 2000 μmol m^{-2} s^{-1} in both types of plants (*in vitro* raised from the second crop shoots and *ex vitro*). The temperature optima for photosynthesis was observed at 25 °C for both *ex vitro* (Figure 3A) and *in vitro* raised (Figure 3B) plants. Maximum rate of photosynthesis was 12.24 μmol m^{-2} s^{-1} in *ex vitro* plants (Figure 3A), and slightly lower (11.95 μmol m^{-2} s^{-1}) in *in vitro* raised plants (Figure 3B) at optimum temperature (25 °C) and optimum light intensity (2000 μmol m^{-2} s^{-1}). It was observed that high light intensity and high temperature together adversely affected CO_2 exchange in this species. The rate of transpiration increased with increasing temperature up to 35 °C irrespective of the light intensity in both *ex vitro* and *in vitro* raised plants; however, net decrease in the rate of transpiration was observed at 40 °C across different light intensities (Figure 3C, D) which

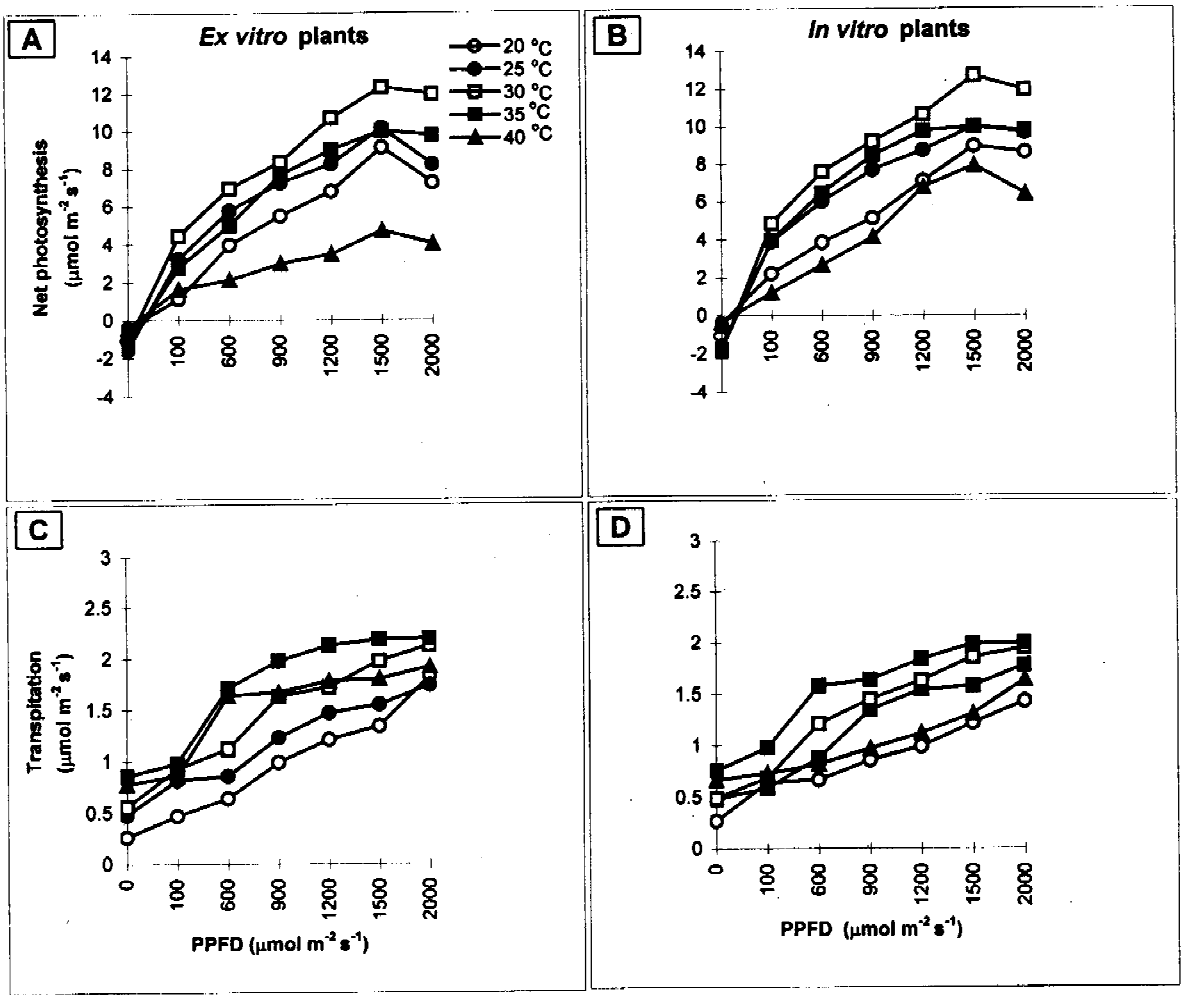

Figure 4. Comparison of rate of gas and water vapour exchange in *ex vitro* (*A* and *C*; seedlings age: 8 months) and *in vitro* raised (*B* and *D*; 8 months following transfer to soil) plants of *Q. glauca* at varying photosynthetic photon flux density (PPFD; 0, 100, 600, 900, 1200, 1500 and 2000 μmol m^{-2} s^{-1}) and temperature (20, 25, 30, 35 and 40 °C). Symbols for different temperatures in Figures *B, C* and *D* are as in Figure *A*.

reflects the closure of stomata due to high temperature stress. In general, differences were statistically not significant as far as the rate of photosynthesis (F-ratio = 0.015, df = 1, p = 0.903) and transpiration (F-ratio = 0.462, df = 1, p = 0.499) of *ex vitro* and *in vitro* raised plants of this species were concerned.

Temperature optima for photosynthesis were observed at 30 °C and light optima were recorded at 1500 μmol m^{-2} s^{-1} in both *ex vitro* and *in vitro* raised plants of *Q. glauca* (Figure 4A, B). Maximum photosynthesis was recorded to be 12.34 and 12.71 μmol $m^{-2}s^{-1}$in *ex vitro* and in *in vitro* raised plants, respectively, at 1500 μmol m^{-2} s^{-1} light intensity and 30 °C; the values decreased at light intensities higher than 1500 μmol m^{-2} s^{-1} in both set of plants. The rate of transpiration increased with higher light intensity irrespective of temperature and was found to be comparable in *ex vitro* and *in vitro* raised plants (Figure 4C, D); the rate was, however, highest (2.20 and 2.00 μmol m^{-2} s^{-1}) at 35 °C and 1500 μmol m^{-2} s^{-1} light intensity in both sets of plants of *Q. glauca*. Differences in the rate of photosynthesis (F-ratio = 2.097, df = 1, p = 0.152) and transpiration (F-ratio = 0.393, df = 1, p = 0.533) of *ex vitro* and *in vitro* raised plants of this species were also statistically not significant.

In general, the higher light and lower temperature optima for photosynthesis recorded in *Q. leucotrichophora* when compared to that of *Q. glauca* indicate that the former should perform better when planted

at exposed sites and higher altitudes. Although the *ex vitro* plants of *Q. leucotrichophora* and *Q. glauca* exhibited slightly higher rates of transpiration and net photosynthesis as compared to *in vitro* raised plants, these differences were statistically not significant. Thus, *in vitro* propagated plants would appear to be quite normal in respect to the physiological functions examined in this study; similar observations have also been reported by Bag et al. (2000) in case of a temperate climate bamboo.

The present study is the first report of *in vitro* propagation of two central Himalayan oak species, namely *Q. leucotrichophora* and *Q. glauca* via multiple shoot formation and subsequent rooting. Using the best protocol (WP medium containing 22.19 μM BA) and cotyledonary nodes, a total of 37 shoots (in *Q. leucotrichophora*) and 22 shoots (in *Q. glauca*) were obtained on per seed basis within a period of 4 months (a total of 4 crops of shoots were harvested). The individual shoots were rooted and the plants transferred to soil. After a period of 1 year, 80 and 70% plants of *Q. leucotrichophora* and *Q. glauca* were found to survive, respectively. Our micropropagation protocol is a useful supplement to the conventional propagation through the establishment of seedling nurseries. When a large number of seeds are used initially, germplasm diversity is maintained and genetic pauperization, often attributed to tissue culture raised 'clonal' plantations is avoided. However, genotypic effects may be a confounding factor and may account for some of the large SE values recorded. This protocol is now being tested on another central Himalayan species of this genus.

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