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Regulation of growth of *Lilium* plantlets in liquid medium by application of paclobutrazol or ancymidol, for its amenability in a bioreactor system: growth parameters

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Abstract Effect of growth retardants (paclobutrazol or ancymidol) was studied in *Lilium* plantlets growing in liquid culture. A significant increase in leaf chlorophyll, epicuticular wax, plant dry weight and bulb starch contents were found in plantlets treated with growth retardants. A similar increase in the number of leaves, roots and bulbs was also noted. However, total leaf area and the fresh weight increased only marginally. These features resulted in robust plantlets that showed significantly improved ex vitro survival. Based on these features, a comprehensive index (CI) was calculated as a measure of quality of the plantlets, and it correlated well with their ex vitro survival. Treatment of plantlets with 3.4 μM paclobutrazol was found to be the best and its carry over effects were also minimal.

Keywords Growth retardants · Paclobutrazol · Ancymidol · Micropropagation · 6-Benzyladenine · Liquid culture · Chlorophyll · Epicuticular wax

Abbreviations ANC: Ancymidol · BA: 6-Benzyladenine · CI: Comprehensive index · GA: Gibberellic acid · PBZ: Paclobutrazol · Rt#: Number of roots · TBV: Total bulb volume · TLA: Total leaf area

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Introduction

Lilies are among the top 10 commercial flowers of the world owing to their large and charming blooms in attractive colours and having excellent vase life. Their in vitro culture has become an excellent tool for the rapid propagation of new and desirable clones for commercial marketing (Lim et al. 1998a). Bulblets produced in vitro, have certain properties that make them ideal propagules. These can be easily handled, transported, stored and are pathogen free if cultures are initiated from pathogen-free materials (Lian et al. 2003). Efforts to scale up their micropropagation using bioreactors and automation in order to achieve commercial competitiveness, essentially requires use of liquid medium (Aitken-Christie and Davies 1988; Ziv 1991, 1992). Though growth and proliferation rates are much higher in liquid medium, the plants suffer from several drawbacks associated with liquid culture (Hazarika 2003; Seon et al. 2000). However, such problems have been effectively controlled by use of growth retardants in several plants such as *Narcissus* (Chen and Ziv 2001), cucumber (Konstas and Kintzios 2003), chrysanthemum and rose (Roberts et al. 1992).

Beneficial effects of triazole in maintaining water balance in draught-stressed plants under in vivo conditions (Davies et al. 1988) and also under in vitro conditions in chrysanthemum (Smith et al. 1990) is well documented. Plant growth retardants (especially inhibitors of gibberellin biosynthesis) are known to reduce the shoot length by shortening the internodes of plants without changing their developmental patterns or being phytotoxic. Besides, they also reduce leaf size, enhance chlorophyll and cause thickening of roots (Smith et al. 1990). They are also known to improve stomatal regulation and reduction in the stomatal aperture possibly due to a general reduction in cell expansion and lowering the rate of cell division, caused by anti-gibberellin activity. This along with epicuticular wax deposition, prevents dehydration upon ex vitro transfer (Smith et al. 1990). Treatments with paclobutrazol resulted in a shift in the partitioning of assimilates from the leaves to the storage organs

and roots, increased carbohydrates in all parts of seedlings, increased chlorophyll, soluble protein and mineral contents in leaf tissues, increased root respiration, reduced cell-wall polysaccharides and water loss (Hazarika 2003). The significance of good bulb formation with high nutrient reserve for ex vitro survival of *Lilium* plants has already been emphasized (Haruki et al. 1998) while, Wang et al. (1999) reported that paclobutrazol-treated *Lilium* plantlets showed better bulb formation, resulting in greater percentage of survival ex vitro. Of the various growth retardants tried, ANC and PBZ were found to be the best for cluster formation in liquid media and also enhance formation of storage organ in many geophytes (Ziv 1990; Ilan et al. 1995). In the present study, the role of ancymidol and paclobutrazol in modulation of the various growth parameters was investigated in order to facilitate liquid culture of *Lilium* plantlets and make it more amenable to culture in bioreactor systems in subsequent studies.

Materials and methods

The explants for experimental work were taken from the aseptic cultures of Asiatic lilies (*Lilium longiflorum* var. Pollyanna) grown in our laboratory. The cultures were being maintained on MS (Murashige and Skoog 1962) medium supplemented with BA (5 μM), IBA (10 μM), NAA (2.7 μM), sucrose (3%, w/v) and agar (0.8%, w/v) for solidification and pH adjusted to 5.8 prior to autoclaving. Cultures were incubated at 25±2°C under a photosynthetic photon flux density (PPFD) of 70±5 μmol m^{-2} s^{-1} from cool white fluorescent lamps. Day length was maintained at 14 h in a 24 h light/dark cycle. The cultures were subcultured every 4 weeks onto fresh medium. The explants used in various experiments, comprised of a small cluster consisting of one to three bulblets, and weighing about 1 g.

Media and growth regulators for experimentation

Liquid MS media with the following combinations of the plant growth regulators were used:

C	MS + NAA 0.54 μM (control)
B_1	control + BA 2.22 μM
B_2	control + BA 4.44 μM
B_3	control + BA 11.1 μM
B_4	control + BA 22.2 μM
P_1	control + PBZ 1.7 μM
P_2	control + PBZ 3.4 μM
P_3	control + PBZ 8.5 μM
P_4	control + PBZ 17 μM
A_1	control + ANC 1.7 μM
A_2	control + ANC 3.4 μM
A_3	control + ANC 8.5 μM
A_4	control + ANC 17 μM
BP_1	control + BA 2.22 μM + PBZ 1.7 μM
BP_2	control + BA 4.44 μM + PBZ 3.4 μM
BP_3	control + BA 11.1 μM + PBZ 8.5 μM
BP_4	control + BA 22.2 μM + PBZ 17 μM

Of these, 'C' was the control, and apart from that, four experimental series 'B', 'P', 'A' and 'BP' were prepared with different concentrations of BA, PBZ, ANC and BA together with PBZ, respectively. Since some preliminary studies indicated the superiority of PBZ treatment, its effect was also studied in conjunction with BA. After inoculation, the cultures were incubated under conditions as described earlier. Data for leaf number, total leaf area, number of roots, number and total volume of bulbs, and fresh weight was recorded on days 0 and 40 and percent increase in each of the parameters was calculated.

For making measurements of total leaf area in in vitro plants, the length and breadth of the leaves were measured aseptically, their area were calculated and summed up for all the leaves on each of the explant cluster. However, for making measurements of leaf area in harvested plants, leaf area meter (CI-203 Area Meter, CID, Inc. USA) was used. The diameter of the bulblets was measured and radius (r) derived. Putting the value of r in the formula $\frac{4}{3}\pi r^3$, the volume of the bulblets was calculated.

Fresh weight of the plant material was measured immediately after harvest and the dry weight was determined after drying at 80°C for 72 h in an electric oven till constant weight was obtained.

For anatomical studies, middle one-third of the fully opened leaves (third) were selected and free-hand transverse sections were cut. These were viewed under a light microscope and photographed with the help of Nikon digital camera DXM-1200 mounted on the microscope.

Chlorophyll estimations were done following method described by Lichtenthaler (1987). Tissues (100 mg fresh weight) of the samples were thoroughly crushed in mortar and pestle and extracted with 10 ml of chilled 80% acetone for 30 min in dark and centrifuged at 10,000 rpm for 5 min at 4°C. The absorbance of the supernatant was determined spectrophotometrically at 645 and 663 nm.

For starch estimation, the sample (0.5 g fresh weight) was homogenized in 10 ml of 80% hot ethanol to remove sugars. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. The residue, after repeated washing with 80% ethanol, was used for starch estimation (McRae 1971) using Anthrone reagent. The intensity of the green colour was read at 630 nm.

Wax content was estimated according to Barnes et al. (1996) using modified procedure described by Pandey and Nagar (2002).

Ex vitro transfer

The plantlets generated through various experiments in in vitro were washed gently, their dead tissues removed, and then transferred to moistened sand bed in a polytunnnel having humidity (78±5%) and temperature 25±5°C. Their survival percentage was calculated after 40 days of transfer.

Table 1. Calculation of comprehensive index (CI) on the basis of absolute indices calculated for various growth parameters

PGRs (μM)	TLA	New rts	Lf#	Starch	DW	Chl	FW	Wax	Blb #	Blb size	Mean
MS	9.3	3.9	8.9	2.9	4.7	6.9	9.0	5.3	6.8	8.2	6.6
BA 2.22	10.0	3.0	10.0	3.8	3.2	4.8	9.9	5.8	9.0	7.7	6.7
BA 4.44	9.0	2.5	9.5	3.3	4.3	5.4	9.1	6.6	8.7	7.3	6.6
BA 11.1	7.6	1.7	7.9	3.5	4.6	5.2	9.5	6.2	8.1	8.2	6.2
BA 22.2	7.0	0.7	7.8	2.8	4.1	4.3	9.9	6.8	6.3	9.2	5.9
PBZ 1.7	8.9	4.7	8.6	10.0	6.5	9.8	10.0	8.2	10.0	7.7	8.4
PBZ 3.4	8.0	9.0	8.0	9.7	7.6	10.0	8.2	8.7	7.2	9.4	8.6
PBZ 8.5	2.4	7.1	4.0	9.3	10.0	9.0	6.9	9.1	7.9	9.0	7.5
PBZ 17	1.3	6.1	3.4	10.0	8.7	7.0	5.8	9.7	7.4	8.3	6.8
ANC 1.7	4.9	6.6	6.3	7.7	6.8	9.2	8.0	8.5	7.8	8.8	7.5
ANC 3.4	5.3	10.0	6.5	8.8	6.4	8.1	8.3	8.5	7.5	9.4	7.9
ANC 8.5	4.4	6.8	6.1	8.8	9.1	6.1	7.2	10.0	7.4	9.3	7.5
ANC 17	3.1	5.0	4.9	9.5	9.1	6.3	6.0	9.1	8.3	9.1	7.0
BA 2.22 + PBZ 1.7	5.9	6.6	7.2	9.5	8.1	7.3	9.0	8.0	8.3	8.5	7.8
BA 4.44 + PBZ 3.4	4.2	2.9	4.7	4.5	7.8	8.2	6.6	7.1	6.9	9.0	6.2
BA 11.1 + PBZ 8.5	2.2	1.6	2.4	3.7	6.0	6.8	6.7	6.8	4.8	10.0	5.1
BA 22.2 + PBZ 17	−0.3	0.2	0.7	3.7	5.8	3.2	5.7	6.9	5.2	9.5	4.0

Blb#, number of bulblets. Blb size, size of bulblets. Chl, chlorophyll. DW, percentage of dry weight. FW, fresh weight. Lf#, number of leaves. rts, roots. TLA, total leaf area.

Some of the plantlets from each of the treatments were transferred to the nutrient medium with control composition. After 30 days of culture, their growth parameters, i.e., number of leaves, total leaf area, chlorophyll content, number of roots, number and size of bulbs, starch content of bulbs and fresh weight of entire plants were determined to observe the after effects of various treatments with any of the growth retardants.

Statistical analysis

Differences in different growth parameters as influenced by different treatments were analysed using one-way ANOVA (analysis of variance) as per Gomez and Gomez (1984) and differences among mean values were tested against critical difference at $P<0.05$.

Calculation of comprehensive index (CI)

For each growth parameter, the treatment was identified for its maximum value. All the values obtained in different treatments were divided by this value and multiplied by 10 to obtain indices on a scale of 10. Further, for each treatment, the mean of these index values for all the growth parameters, was calculated. These were the comprehensive indices (CI) for the treatments, as they were based on all the growth parameters (Table 1). Later, these indices for all the treatments were compared to growth and survival of the respective plantlets upon ex vitro transfer. Coefficient of correlation was calculated between the comprehensive index (CI) and survival percentage, and analysed statistically. All the treatments were arranged in descending order of CI to sort them in order of decreasing suitability for culture of *Lilium* plantlets and hence, identify the most suitable treatment.

Results

For both ANC and PBZ, best growth characteristics were obtained at 1.7 and 3.4 μM, and the observations for the various growth parameters of the plantlets and their ex vitro survival after 40 days of culture are presented in Fig. 1. Mean growth behaviour in response to various treatments are shown in Plate 1.

Effect on average number of leaves per plant cluster

The percent increase in the number of leaves per plant cluster was reduced slightly by application of growth retardants at lower concentrations P_1, P_2, A_1, A_2, A_3, BP_1, but decreased significantly with increase in their concentrations P_3, P_4, A_4, BP_2, BP_3, BP_4. Thus, usage of growth retardants at low concentrations (1.7 and 3.4 μM) did not cause any adverse effects on the leaf formation in in vitro plants (Fig. 1A).

Effect on total leaf area per plantlet cluster

Percent increase in the TLA of the plantlets was significantly reduced by the application of growth retardants, except for PBZ applied alone at low concentrations (P_1 and P_2). This indicated that whereas PBZ could be used at low concentrations without having any adverse effect on leaf growth, ANC adversely affected leaf growth at all the concentrations (Fig. 1B).

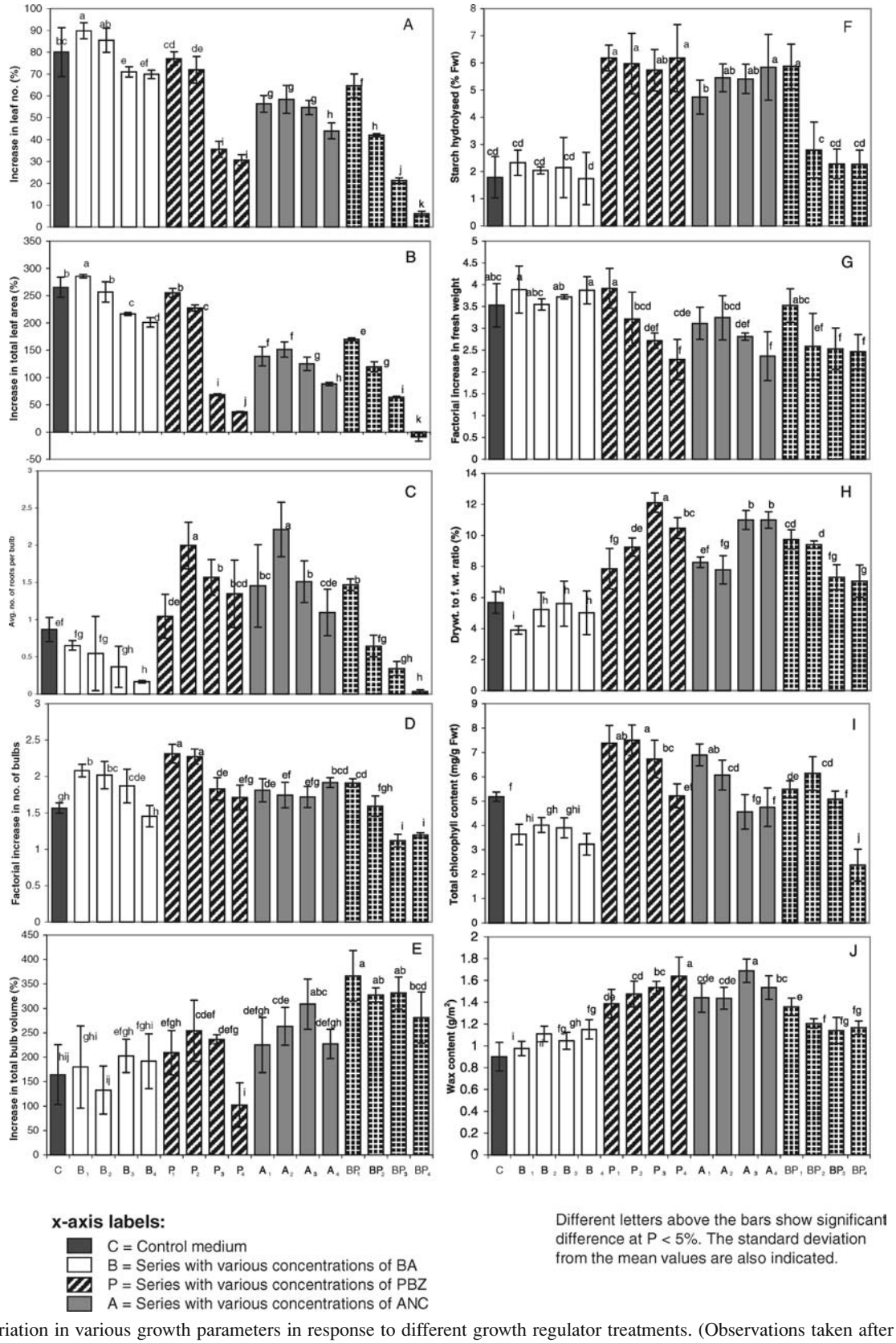

Fig. 1 Variation in various growth parameters in response to different growth regulator treatments. (Observations taken after 40 days of treatment)

Plate 1 Comparative growth of plantlets under different growth regulator treatments at different concentrations, after 40 days. (**A**) Clockwise: C, B_1, B_2, B_3, B_4. (**B**) Clockwise: C, A_1, A_2, A_3, A_4. (**C**) Clockwise: C, P_1, P_2, P_3, P_4. (**D**) Clockwise: C, BP_1, BP_2, BP_3, BP_4

Effect on total number of roots per bulb

Treatment of plantlets with growth retardants significantly promoted the number of roots per bulblet. Roots showed prolific growth and remained normal at low concentrations of growth retardants (P_1, P_2, A_1 and A_2), but these turned stout and swollen at higher doses (P_3, P_4, A_3 and A_4) (Fig. 1C; Plate 1).

Effect on average number of bulblets per plantlet cluster

Growth retardants as well as BAP, had a similar effect on factorial increase in the average number of bulblets. Both promoted it at low concentrations, when applied separately. This indicated that use of growth retardants alone at low concentrations (1.7 and 3.4 μM) was sufficient to induce proliferation (Fig. 1D).

Effect on total bulb volume

A significant increase in TBV was observed in the presence of growth retardants at concentrations of 1.7, 3.4, 8.5 μM. Unlike the effect of plant growth regulators on TLA and Rt#, a significant increase in TBV was observed even in the presence of BA along with PBZ, the highest being 366% for BP_1 (BA 2.22 μM + PBZ 1.7 μM) (Fig. 1E).

Effect on starch content in bulblets

The presence of growth retardants significantly promoted starch content of the bulbs except at higher concentrations especially in the simultaneous presence of BA (BP_2, BP_3, BP_4). This was also evident by their swollen and nodular appearance (Plate 2D and E). Starch accumulation was comparable for both the growth retardants presently employed and for all the concentrations tested, without much variation (Fig. 1F).

Effect on increase in total fresh weight

No significant difference in increase of fresh weight was observed in the presence of growth retardants at low concentrations (P_1, P_2, A_1, A_2 and BP_1) as compared to control. However, there was a proportionate decrease in fresh weight with increase in their concentrations (P_3, P_4, A_3, A_4, BP_2, BP_3 and BP_4) (Fig. 1G).

Effect on ratio of dry weight to fresh weight

The percentage dry weight content in the plantlets enhanced significantly in response to treatment with growth retardants as compared to those on control medium or the ones grown on BA-supplemented media. Moreover, it increased with increase in concentration of the growth retardants. However, when PBZ was used in combination with BA, it showed a steady decline with increasing concentrations (Fig. 1H). This implied that use of growth retardants alone was sufficient for increasing the dry weight percentage of the *Lilium* plantlets.

Effect on chlorophyll content

The total chlorophyll content of leaves showed a significant increase when plantlets were cultured in the presence of

Plate 2 (**A**) Plantlets growing in B_2 medium after 15 days. (**B**) Plantlets growing in P_2 medium after 15 days. (**C**) Plantlets after 40 days culture on B_2 medium. (**D**) Plantlets after 40 days culture on P_2 medium. (**E**) Plantlets after 40 days culture on P_4 medium

growth retardants especially at lower concentrations. Presence of BA along with PBZ did not change this effect significantly, except for the highest concentration tested (BP_4). Treatment with BA alone resulted in poor development of chlorophyll, as compared to control (Fig. 1I). Hence, application of both PBZ or ANC at 1.7 or 3.4 μM was suitable for generation of plants with higher chlorophyll content in their leaves, which is important for attaining autotrophy on ex vitro transfer.

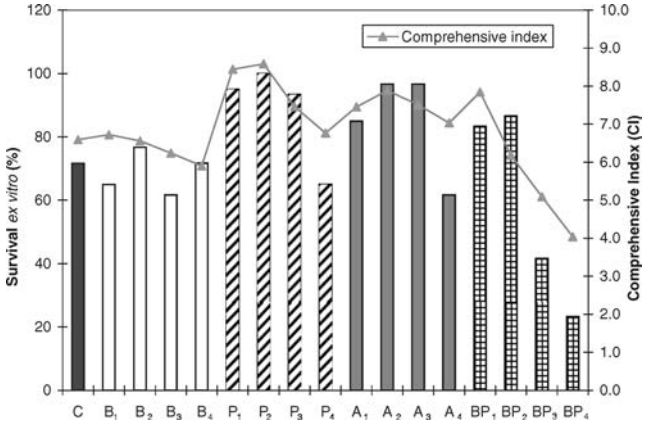

Fig. 2 Comparison of the survival percentage upon 40 days of ex vitro transfer and the calculated comprehensive index, for plantlets raised with various growth regulator treatments at different concentrations

Effect on cuticular wax content

The total cuticular wax content of the leaves increased significantly with all the treatments as compared to control. However, with the application of both the growth retardants, the contents were higher than those treated with BA alone. Among various treatments, P_4 and A_3 had the maximum epicuticular wax contents (Fig. 1J).

Effect on ex vitro survival of plantlets

On 40 days of ex vitro transfer, the plants treated with growth retardants in the preceding media (especially P_1, P_2, P_3, A_1, A_2, A_3, BP_1, BP_2), exhibited better survival and better growth. Maximum survival percentage (100%) was observed for plantlets raised on medium with 3.4 μM PBZ (P_2) alone and was found to be the best for generation

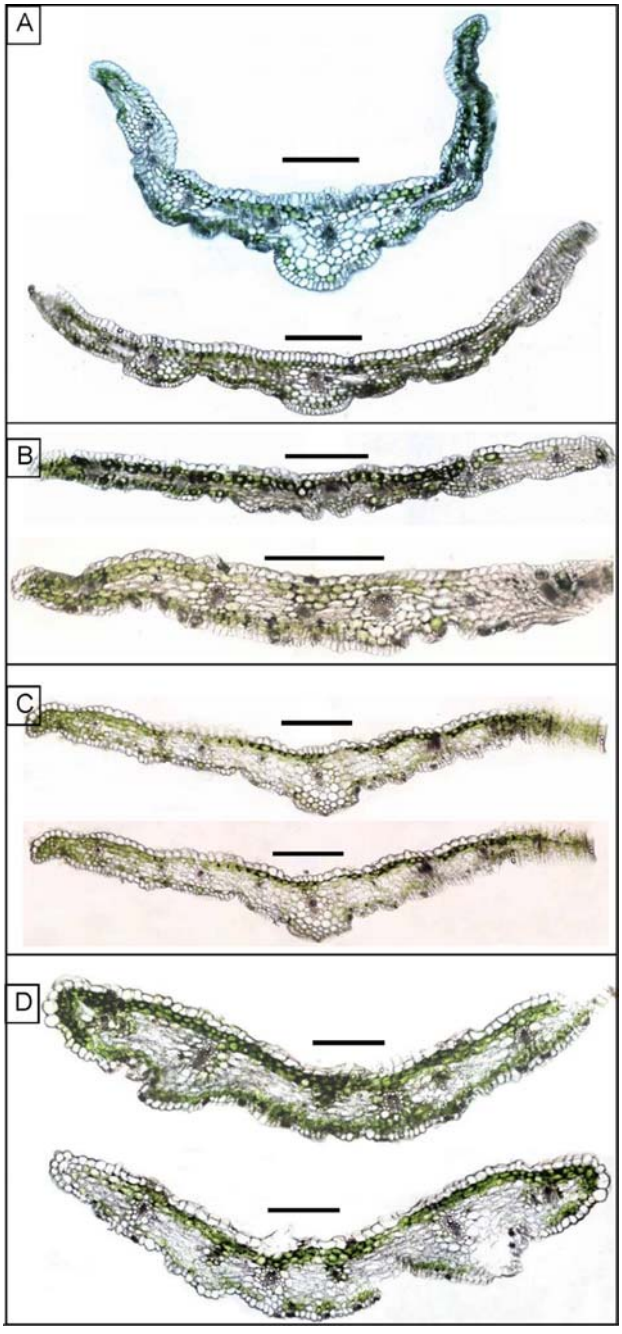

Plate 3 Anatomical details of leaves obtained from plantlets cultured on different media. (**A**) MS + NAA 0.54 μM + BA 2.22 μM. (**B**) MS + NAA 0.54 μM + PBZ 3.4 μM. (**C**) MS + NAA 0.54 μM + ANC 3.4 μM. (**D**) MS + NAA 0.54 μM + BA 11.1 μM + PBZ 8.5 μM. Deformities were observed only in simultaneous presence of BA and PBZ at higher concentrations (**D**). (Bar: 500 μm)

of quality plantlets showing better adaptation and highest survival on ex vitro transfer (Fig. 2).

Comprehensive index (CI) in relation to survival of plants ex vitro

Indices normalized to a scale of 10, for each growth parameter in all the treatments, and calculation of the comprehensive growth index (CI) for all the treatments are summarized in Table 1. The CIs for all the treatments showed a strong correlation of 0.89 with the respective actual survival of the plantlets upon ex vitro transfer (Fig. 2) and this was significant at $P \leq 1\%$. Based on it, reasonably accurate predictions for ex vitro survival of plantlets could be made.

Also based on the CI and the actual survival percentage upon ex vitro transfer, 3.4 μM PBZ (P_2) treatment was found to be the best for generating robust *Lilium* plantlets.

Anatomical studies

There were no significant differences between the anatomical characteristics of leaves of plantlets treated with BA, PBZ or ANC alone (Plate 3A–C). However, deformities were observed in leaves from plantlets cultured in the presence of higher PBZ (8.5 μM) along with BA (11.1 μM) (BP_3) (Plate 3D).

In vitro performance of growth–retardant treated plantlets upon transfer to growth–retardant-free medium

Prior treatment with growth retardants P_1, P_2, P_3, P_4, A_1, A_2 resulted in production of plantlets with numerous leaves (Fig. 3A) but less leaf area (Fig. 3B). Most remarkable was the pattern of total chlorophyll content in leaves, which was higher in all the plantlets treated prior with growth retardants at all the concentrations (Fig. 3B). The number of new roots per cluster increased proportionally with the concentrations of the growth retardant in the treatment in the preceding culture media upto 8.5 μM (P_1, P_2, P_3, A_1, A_2 and A_3), but showed a decrease at 17 μM (P_4 and A_4) (Fig. 3C). Differences in percent increase in fresh weight, factorial increase in number of bulblets and TBV, though not clearly distinct, showed a marginal increase for various growth retardant concentrations (Fig. 3D and F). Though plantlets from medium with BA exhibited a comparable increase in TBV, their bulbs were less nodular as compared to the ones earlier raised on medium supplemented with growth retardants. The increase was more due to swelling of the leaf bases with water than because of dry matter accumulation. This was confirmed by the analysis of starch content in the bulbs, which showed a remarkable increase with an increase in the growth retardant concentrations in the preceding culture media (Fig. 3F).

Discussions

In the present study, the development of a protocol incorporating growth retardants (ANC and PBZ) for efficient micropropagation of *Lilium* plantlets, optimised for liquid system has been achieved, which is, amenable to a bioreactor system (studies to be reported later).

Application of ANC or PBZ at 1.7 or 3.4 μM facilitated development of plantlets with higher bulb-to-leaf ratio.

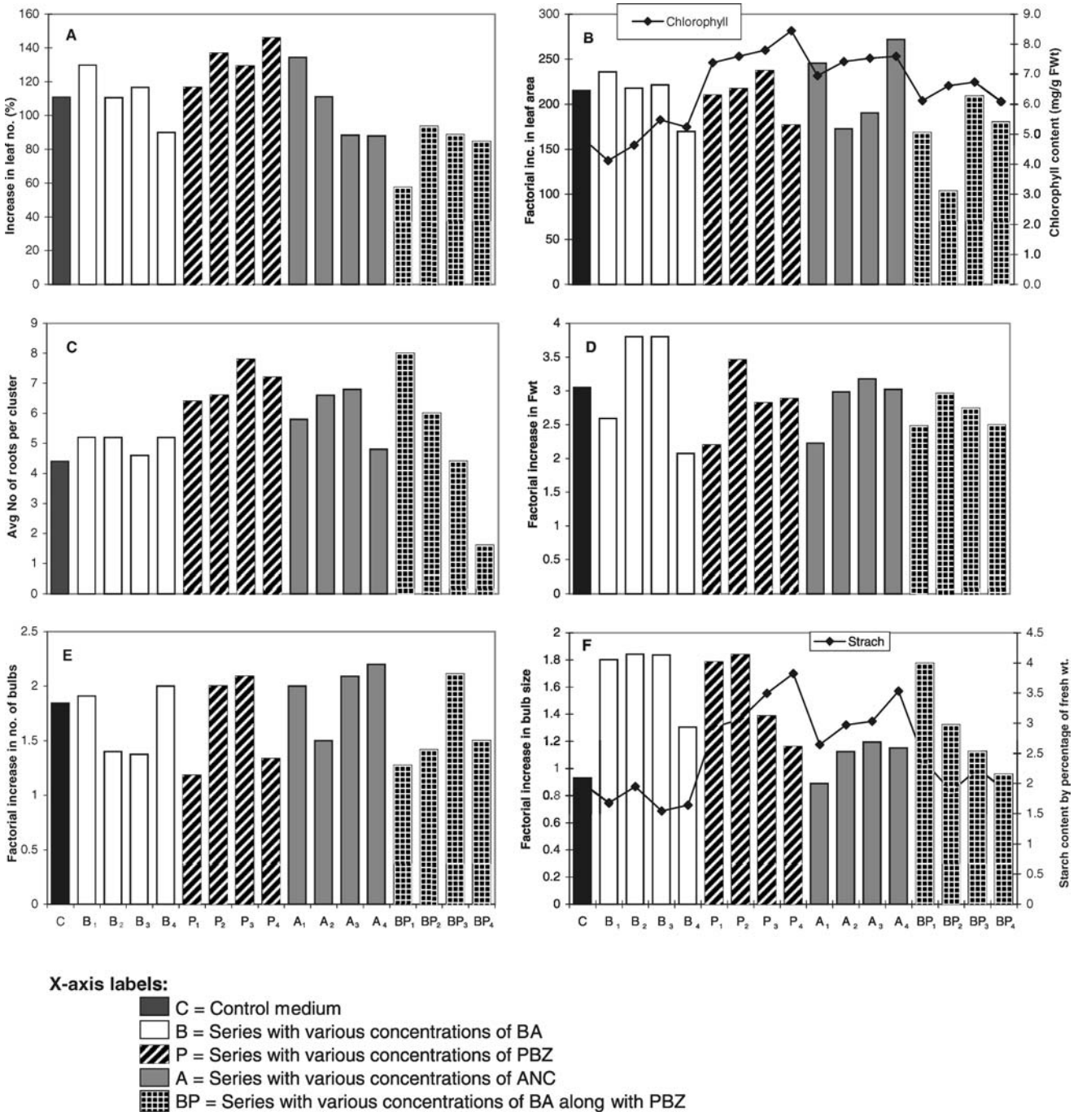

Fig. 3 Variation in various growth parameters in response to different growth regulator treatments in the preceding culture. (Observations taken after 30 days of transfer to growth retardant free medium.) (**A**) Increase in leaf number (%); (**B**) Factorial increase in leaf area; (**C**) Average number of roots per cluster; (**D**) Factorial increase in fresh weight; (**E**) Factorial increase in number of bulbs; (**F**) Factorial increase in bulb size

Treated plantlets had larger nodular bulbs with higher starch content and also reduced size leaves as compared to the untreated as well as BA-treated plantlets (Fig. 1B, E and F). However, their rate of proliferation remained unaffected as compared to that of plantlets treated with BA (Fig. 1D). This is in accordance with some other studies like in *Philodendron* (Ziv and Ariel 1991), *Gladiolus* (Ziv 1989; Steinitz and Lilien Kipnis 1989) and *Nerine* (Ziv 1992), where similar role of ANC and PBZ has been described for promotion of profuse shoot proliferation in the form of clusters in liquid system. Better acclimatization ability of plantlets treated with growth retardants was suggested to be due to larger bulb size, which enhanced chances of leaf emergence after transplantation (Lian et al. 2003). Further, reduction in shoot growth was also reported in response to PBZ treatment in apple (Quinlan

and Richardson 1984; Steffens et al. 1985). Interestingly, Lim et al. (1998b) in their study found that most of the growth retardants did not have any favourable impact on bulb development in four cultivars of lilies, except with B_9 (daminozide) at higher concentrations. However, in the present study, bulb formation was significantly promoted by both the growth retardants, PBZ or ANC even at lower concentrations (1.7 and 3.4 μM) (Plate 1B–D).

Both PBZ and ANC at optimum concentrations (1.7 and 3.4 μM) also stimulated formation of numerous normal roots without the application of any other growth regulator, and at higher concentrations (8.5 and 17 μM), an unusual thickening of roots was observed (data not shown). The contents of dry weight, chlorophyll and epicuticular wax also showed significant increase (Fig. 1H–J). All these improved features conferred robustness to the plantlets, ensuring their survival upon ex vitro transfer. Similarly, Smith et al. (1990, 1992) in response to PBZ treatment in chrysanthemum and grapevine, reported shortened stems, thickened roots, increased chlorophyll concentration per unit area of leaf, increased epicuticular wax, normal stomatal functioning and reduced stomatal apertures. It was suggested that these features facilitated development of hardy plants, which showed reduction in wilting after ex vitro transfer. This also finds support from the studies of Ziv (1992) and Ziv and Ariel (1991) in *Philodendron*.

The increase in chlorophyll content by PBZ treatment could be explained on the basis of two facts: First, that the leaves of both treated and untreated plants may contain the same number of cells, but because the cells in treated ones are generally smaller, the chlorophyll is more concentrated inside the reduced cell volume. Second, there is also evidence (Dalziel and Lawrence 1984) that the amount of chlorophyll is actually increased due to increase in phytyl (an essential part of chlorophyll molecule), which is synthesized via the same terpenoid pathway as gibberellins.

The shortening of the plantlets and thickening of roots in the present study in response to growth retardant treatment could be explained on the basis that these compounds block separate steps in the biosynthetic pathway of GAs, which stimulate cell elongation. Once GA production is inhibited, cell division still occurs, but the new cells do not elongate. It is also possible that the activity of meristematic areas is influenced and the subapical meristems which are responsible for elongation get affected. Based on histological evidences in soybean and maize seedlings, Nitsche et al. (1985) reported that at low concentration of growth retardants, shortening of stem is primarily due to an inhibition of cell elongation, whereas, at higher concentration, the stunting of shoot sections is increasingly attributable to a reduction in cell division.

The comprehensive index (CI) based on the measurements of all the growth parameters, correlated well with the actual ex vitro survival data and served as a reasonably accurate indicator of ex vitro survivability of the plantlets (Fig. 2).

Ziv and Ariel (1991) reported that use of growth retardants has a carryover effect on the bud clusters in the second subculture cycle in growth–retardant-free medium, resulting in prevention of shoot elongation. This necessitates an additional culture period on medium free of growth retardants, thereby increasing the cost and labour associated with plant production (Lorenzo et al. 1998; Ziv and Shemesh 1996). Upon transfer of growth–retardant-treated plantlets to growth–retardant-free medium, the after-effects were not evident for optimal growth retardant concentrations (1.7 and 3.4 μM), but for higher concentrations (8.5 and 17 μM), increases in number of roots per cluster, number of bulbs, TBV and starch content were noticed. Chlorophyll content was, however, higher in the plantlets precultured on medium with growth retardants at all the concentrations tested (Fig. 3B). It could be inferred that, although the carry-over effects of treatment with growth retardants in the preceding cultures were present, these were only beneficial for improvement in quality of plantlets as compared to the ones cultured earlier on medium with or without BA.

Hence, treatment of plantlets with PBZ at 3.4 μM was found to be the most suitable for maintaining proliferating cultures in liquid medium, as well as for generating robust plantlets capable of successful ex vitro transfer. This work comprises an important step towards promotion of culture of *Lilium* plantlets in bioreactor.

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References

Aitken-Christie J, Davies HE (1988) Development of a semi-automated micropropagation system. Acta Hort 230:081–087

Barnes JD, Percy KE, Nigel PD, Jones P, Mclaughlin CK, Mullineaux PM, Creissen G, Wellburn AR (1996) The influence of UV-B radiation on the physiochemical nature of tobacco (*Nicotiana tabacum* L.) leaf surfaces. J Exp Bot 47:99–109

Chen J, Ziv M (2001) The effect of ancymidol on hyperhydricity, regeneration, starch and antioxidant enzymatic activities in liquid-cultured *Narcissus*. Plant Cell Rep 20:22–27

Dalziel J, Lawrence DK (1984) Biochemical and biological effects of kaurene oxidase inhibitors such as paclobutrazol. British Plant Growth Regulatory Group Monograph II, pp 43–57

Davies TD, Steffens GL, Sankhla N (1988) Triazole plant growth regulators. Hort Rev 10:63–105

Gomez KA, Gomez AA (1984) Statistical procedures for agricultural research. Wiley, New York

Haruki K, Hosoki T, Nako Y (1998) Effects of illumination on nutrient absorption from the liquid-shaking medium by enlarging miniature lily bulbs (*Lilium japonicum* Thunb.). J Jpn Soc Hort Sci 67(1):106–111

Hazarika BN (2003) Acclimatization of tissue-cultured plants. Curr Sci 85(12):1704–1712

Ilan A, Ziv M, Halevy AH (1995) Propagation and corm development of Brodiaea in liquid cultures. Sci Hort 63:101–112

Konstas J, Kintzios S (2003) Developing a scale-up system for the micropropagation of cucumber (*Cucumis sativus* L.): the effect of growth retardants, liquid culture and vessel size. Plant Cell Rep 21:538–548

Lian ML, Chakrabarty D, Paek KY (2003) Growth of *Lilium* oriental hybrid `Casablanca' bulblet using bioreactor culture. Sci Hort 97:41–48

Lichtenthaler M (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Lim S, Seon JH, Paek KY, Son SH, Han BH, Drew RA (1998a) Development of pilot scale process for mass production of *Lilium* bulblets *in vitro*. Acta Hort 461:237–241

Lim S, Seon JH, Son SH, Han BH, Paek KY (1998b) Effect of light, inorganic salts and growth retardants on bulblet formation in *Lilium*. J Kor Soc Hort Sci 39(1):107–110

Lorenzo JC, Gonzales BL, Escalona M, Teisson C, Espinosa P, Borroto C (1998) Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tiss Org Cult 54(3):197–200

McRae JC (1971) Quantitative measurement of starch in very small amount of leaf tissue. Planta 96:101–104

Murashige T, Skoog FA (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–479

Nitsche K, Grossmann K, Saurebrey E, Jung J (1985) Influence of the growth retardant tetcyclacis on cell division and cell elongation in plants and cell cultures of sunflower, soybean, and maize. J Plant Physiol 118:209–218

Pandey S, Nagar PK (2002) Leaf surface wetness and morphological characteristics of *Valeriana jatamansi* grown under open and shade habitat. Biol Plant 45:291–294

Quinlan JD, Richardson PJ (1984) Effect of paclobutrazol (PP333) on apple shoot growth. Acta Hort 146:105–110

Roberts AV, Walker S, Horan I, Smith EF, Mottley J (1992) The effects of growth retardants, humidity and lighting at stage III on stage IV of micropropagation in chrysanthemum and rose. Acta Hort 319:153–157

Seon JH, Kim YS, Paek KY (2000) The fed-batch culture system using bioreactor for the bulblets production of ornamental lilies. Acta Hort 520:53–59

Smith EF, Gribaudo I, Roberts AV, Mottley J (1992) Paclobutrazol and reduced humidity improve resistance to wilting of micropropagated grapevine. Hort Sci 27:111–113

Smith EF, Roberts AV, Mottley J (1990) The preparation *in vitro* of chrysanthemum for transplantation to soil. 2. Improved resistance to desiccation conferred by paclobutrazol. Plant Cell Tiss Organ Cult 21:133–140

Steffens GL, Wang SY, Faust M, Byun JK (1985) Controlling plant growth via the gibberellin biosynthesis system. I. Growth parameter alterations in apple seedlings. Physiol Plant 63:163–168

Steinitz B, Lilien Kipnis H (1989) Control of precocious gladiolus corm and cormel formation in tissue culture. J Plant Physiol 135:477–484

Wang AQ, He LF, Zhou Q, Sheng YP (1999) Comparative study on transplanting of lily plantlets. J Guangxi Agric Biol Sci 18(3):187–189

Ziv M (1989) Enhanced shoot and cormlet proliferation in liquid cultured gladiolus buds by growth retardants. Plant Cell Tiss Org Cult 17:101–110

Ziv M (1990) The effect of growth retardants on shoot proliferation and morphogenesis in liquid cultured gladiolus plants. Acta Hort 280:207–215

Ziv M (1991) Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh PC, Zimmerman RH (eds) Micropropagation: technology and Application. Kluwer Academic Publishers, Dordrecht, pp 45–69

Ziv M (1992) Morphogenic control of plants micropropagated in bioreactor cultures and its possible impact on acclimatization. Acta Hort 319:119–124

Ziv M, Ariel T (1991) Bud proliferation and plant regeneration in liquid-cultured philodendron treated with ancymidol and paclobutrazol. J Plant Growth Regulat 10(1):53–57

Ziv M, Shemesh D (1996) Propagation and tuberization of potato bud clusters from bioreactor culture. *In vitro* Cell Dev Biol Plant 32:31–36