ABSTRACT

Brassinosteroids are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses including somatic embryogenesis in conifers. This study highlights the successful brassinolide-mediated stimulation of embryogenesis in all the five genotypes of Pinus caribaea tested. 24-epiBrassinolide at 2.0 μM with 9.0 μM 2, 4-dichlorophenoxy acetic acid enhanced the formation of embryogenetic tissue from mature zygotic embryos on half-strength MSG basal medium. However, the frequency of somatic embryogenesis was not similar in all the five genotypes tested. The highest percentage of somatic embryogenesis (87.0 ± 1.8) was recorded in PC 05 genotype. On the other hand the lowest percentage of somatic embryogenesis (70.0 ± 1.7) was obtained in PC11 genotype. The developed somatic embryos on maturation medium after 12 to 14 weeks in all the five genotypes tested. Therefore, 24-epiBrassinolide can be used as growth regulator in conifer somatic embryogenesis for improving the initiation of embryogenic cultures of recalcitrant pines for the commercial forestry programmes particularly in Indian subcontinent.

Keywords: cell suspension, India, Pinus caribaea, somatic embryogenesis, tropical pine

INTRODUCTION

Conifers are an integral part of human life, and a vital component of biodiversity. Conifers in particular are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. The global demand for wood is not expected to decrease in the near future; in fact, the demand for several wood products is more likely to increase. To lower the pressure on existing forests, mainly rain and conifer forests, a global effort is very much needed to include trees in the modern era of plant breeding. Plantation forestry, with optimized and increased forest productivity, is likely to become the major source for wood products in the near future (Giri 2007c; Malabadi 2008a, 2008b). Efficient and inexpensive large-scale clonal propagation of superior clones, are key elements for the successful reforestation and management of future commercial forests. To maintain and sustain forest vegetation, conventional approaches have been exploited in the past for propagation and improvement. However, such efforts are confronted with several inherent bottlenecks. Currently, most tree-improvement programs with biotechnological approach are based on the selection of superior clones from existing forests, and used for clonal propagation of the selected clones using tissue culture techniques. Plant tissue culture techniques viz somatic embryogenesis exploits the capacity for in vitro regeneration (Malabadi et al. 2009a, 2009c). Somatic embryogenesis can be defined as the formation of an embryo from a cell other than a gamete or the product of gametic fusion (Konar and Nataraja 1965; Nataraja and Konar 1970). This approach appears to have several advantages over other in vitro propagation systems, including its potentially high multiplication rates, potential for scale up and delivery via bioreactor and synthetic seed technologies, and in fact that embryogenic cultures make suitable target tissue for gene transfer (Malabadi and Nataraja 2003; Malabadi and van Staden 2005b; Malabadi and Nataraja 2006, 2007c; 2007d; Malabadi et al. 2008a, 2008b, 2008c, 2008d, 2008e). Typically, conifer somatic embryogenesis proceeds through four steps; initiation, maintenance, maturation, germination, regeneration of plantlets, and cryopreservation of embryogenic tissue could be added as a final step when storage is needed (Malabadi and Nataraja 2006).

Of the coniferous trees, the genus Pinus is found to be most important, has the largest distribution, and is found in most diverse climates. Pinus caribaea Mor. (Caribbean pine) is one of the tropical pines that grow naturally at low altitudes. Its rapid growth and high-quality wood that is hard and resistant makes it a useful tree for pulp, carton, paper and veneer production. Consequently, it has become one of the most important Pinaceae for forestry use in tropical and subtropical areas (David et al. 1995). As an exotic species, P. caribaea has shown great promise in India for its fast growth and suitability for planting in low altitudes (Nadguda et al. 1993). Somatic embryogenesis was obtained from cultured immature embryos of P. caribaea Morelet var. Hondurensis on a modified Hakman and von Arnold (1985) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), N\(^6\)-benzyladenine (BA) and/or kinetin. Embryogenic calli could be subcultured for more than 10 months and retain their embryogenic ability in P. caribaea (Laine and David 1990). Embryos bearing well differentiated cotyledons were recovered after transfer of calli to modified Hakman and von Arnold (1985) medium supplemented with abscisic acid and devoid of organic nitrogen. Rooted plantlets in P. caribaea were obtained after transfer to a hormone-free medium. Protoplasts were isolated from embryogenic cells suspensions of P. caribaea. Sustained divisions of protoplast-derived cells and regeneration of somatic pro-embryos were achieved using the medium designed for the culture of cotyledon protoplasts of P. caribaea, and then dilution with the cell suspension medium. The appearance and nutritional requirements of the established protoplast-derived cell suspension were similar to those of the original cell suspension. Cell aggregates were transferred to solid medium for callus development. Fully differentiated embryos with cotyledons and a hypocotyl
were obtained 5 months after protoplast isolation in *P. caribaea* (Laine and David 1990). There are no reports of 24-epiBr-Brassinolide (24-epiBr)-induced somatic embryogenesis in *P. caribaea*, and the existing protocols yield a very low percentage of somatic embryos and plantlets too (Laine and David 1990; Nadgauda et al. 1993).

Therefore, the aim of this study was to identify the role of 24-epiBr how different genotypes of *P. caribaea* responsive, and regulate the development of somatic embryos. The present investigation reports the role of 24-epiBr as a growth regulator for the induction of somatic embryos and the regeneration of plantlets using mature zygotic embryos as explants from five genotypes of *P. caribaea*.

**MATERIALS AND METHODS**

**Plant material**

*Pinus caribaea* seeds were collected from five trees (PC05, PC45, PC173, PC12, and PC11) from the Western Ghats Forests of Karnataka state, and from Panchgani (Maharashtra state), a town situated in the south western part of India. Seeds were washed with 1% (v/v) Citramide for 2 min, and rinsed with sterilized distilled water (SDW) three times. Seeds were further treated with sodium hypochlorite solution (4.5% available chlorine) for 2 min, rinsed 5 times with SDW and treated with 0.1% (v/v) Hydrogen peroxide for 24 hr. Immediately prior to excision of embryos, seeds were decontaminated sequentially with 0.1% (v/v) HgCl2 for 2 min, immersed in 70% (v/v) ethanol for 3 min and finally rinsed thoroughly five times with SDW (Malabadi et al. 2005a; Malabadi and Nataraja 2007e).

**Culture medium and initiation of embryogenic tissue**

Mature zygotic embryos were cultured individually on half-strength (inorganic salts) MSG basal medium (Becwar et al. 1990) containing 2.0 g l−1 Gellan gum (Sigma, St. Louis, USA), 90 mM maltose (Hi-media, Mumbai), 1.0 g l−1 L-glutamine, 1.0 g l−1 casein hydrolysate, 0.5 g l−1 nyo-mositol, 0.2 g l−1 p-aminobenzoic acid and 0.1 g l−1 folic acid. 24-epiBr was purchased from CID Tech. Research Inc., Mississauga, Ontario, Canada (www.cidtech-research.com/brass.html). Stock solutions of 24-epiBr were prepared in absolute ethanol. The medium was supplemented with a range of 24-epiBr concentrations (0.1, 0.5, 1, 2, 5, 10 and 15 μM) and 9.0 μM 2,4-D. The cultures were initiated in 25 mm × 145 mm glass culture tubes (Borosil) with 15 ml medium and maintained in the dark for 4-6 weeks at 25 ± 3°C. Media without 24-epiBr served as the control. The pH of the media was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The pH of the media was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The medium were then sterilized by autoclaving at 121°C at 1.04 Kg cm−2 for 15 min. L-glutamine, p-aminobenzoic, and 24-epiBr were filter sterilized (Whatman filter paper, pore size = 0.45 μm; diameter of paper = 25 mm), and added to the media after it had cooled to below 50°C.

All the cultures were examined for the presence of embryonal suspensor masses by morphological and cytological observations of callus. The cultures showing white muclaginous embryogenic tissue were identified and subcultured on the initiation medium for a further 3 weeks for the improved development of embryonal suspensor masses. Half-strength (inorganic salts) MSG medium supplemented with 9.0 μM 2, 4-D and 2.0 μM 24-epiBr was used as an initiation medium for this purpose.

**Maintenance of embryogenic tissue**

The white muclaginous embryogenic tissue developed on the above initiation medium (I) was subcultured on to maintenance MSG medium (II), containing 130 mM maltose, 4.0 g l−1 Gellan gum, 2 μM 2,4-D and 0.5 μM 24-epiBr. On the maintenance medium, the embryogenic tissue containing embryonal suspensor masses was maintained for 3 weeks with two subcultures. All cultures were maintained in the dark.

**Maturation of somatic embryos**

For maturation, embryogenic tissue clumps of each of the 5 genotypes were incubated in the dark at room temperature (28 ± 2°C). The percentage somatic embryogenesis was calculated as responsive callus-based embryogenesis (expressed in terms of number of responsive regrowth of callus pieces/100 since a total of 100 pieces of calluses were subcultured). Therefore, 5 g of embryogenic tissue of each genotype was aseptically removed and chopped into 100 pieces under aseptic conditions by using a normal scalpel and blade and subcultured on maintenance medium for the growth of callus. The number of pieces re-calling from 100 sub-cultured embryogenic tissues indicates the percentage somatic embryogenesis. This was estimated (i.e. the total number of somatic embryos, germinated embryos, and somatic seedlings produced/g fresh weight (FW) of tissue was calculated) before transferring the embryogenic tissue onto maturation medium for the growth of embryogenic tissue of each genotype was transferred to empty sterile Petri dishes (60 mm diameter) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 h to obtain the desired extent of desiccation. After desiccation, the partially desiccated embryogenic tissues (1 g-pieces × 5/Petri dish) of each genotype were transferred to maturation medium for 2 weeks. After 2 weeks, all the half-strength (inorganic salts) MSG medium supplemented with 180 mM maltose, 120 μM abscisic acid (ABA; Sigma, ACS grade) and 10.0 g l−1 Gellan gum (maturation medium) was used for this purpose. All cultures were placed in the dark at 25 ± 2°C and maintained for 8-12 weeks (Malabadi and Nataraja 2005a, 2007e).

**Germination and plantlet recovery**

After 12 weeks of maturation in the presence of ABA and a higher concentration of maltose (60 g l−1 maltose), the cotyledonal somatic embryos were recovered from the cultures for germination. Before germination, cotyledonal somatic embryos of all 5 genotypes were cold pre-treated at 2°C and kept in the dark for 25 days. The germination medium consisted of half-strength MSG medium with 2 g l−1 Gellan gum (Malabadi and Nataraja 2005a, 2007e). In the first week of germination, cultures were kept in the dark then transferred to diffuse light (30 μmol m−2 s−1) in the second week, and thereafter to a 16-hr photoperiod under a light intensity of 50 μmol m−2 s−1 for hardening. Somatic embryos were considered germinated as soon as radicals elongated while conversion to plantlets was based on the presence of epicotyls. After 4-6 weeks on germination medium, plantlets were transferred to vermiculite in a controlled growth room.

**Statistical analysis**

In above experiments, each replicate contained 50 cultures and one set of experiments consisted of two replicates (i.e. total of 100 cultures per experiment) for each genotype. All the experiments were repeated in triplicate. Data in Tables 1 and 2 represent the average of three independent experiments. Data was arcsine transformed before being analyzed for significance using ANOVA (p<0.05) or evaluated for independence using the Chi-square test. Further, the differences in means were contrasted using Duncan’s multiple range test (DMRT) (α=0.05) following ANOVA. All statistical analyses were performed using SPSS (Microsoft Windows ver. 13.0.1.1) statistical software package.

**RESULTS AND DISCUSSION**

In the present study, mature zygotic embryos cultured on half-strength MSG basal medium containing 9.0 μM 2,4-D without 24-epiBr (control) and 24-epiBr at 0.1 10 and 15 μM produced white, glossy non-embryogenic tissue in all five (PC05, PC45, PC173, PC12, and PC11) *P. caribaea* genotypes. The cultures failed to produce embryonal suspensor masses (ESMs) and ultimately resulted in the browning of tissue and were discarded. On the other hand, mature zygotic embryos produced white muclaginous embryogenic tissue on MSG containing 9.0 μM 2,4-D and 24-
Table 1 The effect of various concentrations of 24-epibrassinolide on the initiation of embryogenic cultures in five genotypes of *Pinus caribaea* cultured on half-strength MSG basal medium containing 9.0 μM 2,4-D.

<table>
<thead>
<tr>
<th>24-epibrassinolide (μM)</th>
<th>Embryogenic tissue initiation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC05</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 ± 0.0 c</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0 ± 0.0 c</td>
</tr>
<tr>
<td>0.5</td>
<td>4.0 ± 0.1 b</td>
</tr>
<tr>
<td>1.0</td>
<td>11.0 ± 0.5 b</td>
</tr>
<tr>
<td>2.0</td>
<td>87.0 ± 2.1 a</td>
</tr>
<tr>
<td>5.0</td>
<td>3.0 ± 0.1 b</td>
</tr>
<tr>
<td>10</td>
<td>0.0 ± 0.0 c</td>
</tr>
<tr>
<td>15</td>
<td>0.0 ± 0.0 c</td>
</tr>
</tbody>
</table>

Table 2 Somatic embryogenesis and seedling recovery in five genotypes of *Pinus caribaea*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Somatic embryogenesis* (%)</th>
<th>Somatic embryos per g fresh wt of embryogenic tissue</th>
<th>Seedlings per g fresh wt of embryogenic tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC05</td>
<td>87.0 ± 1.8 a</td>
<td>30.0 ± 1.0 a</td>
<td>21.0 ± 0.9 a</td>
</tr>
<tr>
<td>PC173</td>
<td>75.0 ± 2.0 a</td>
<td>58.0 ± 3.0 a</td>
<td>45.0 ± 1.8 a</td>
</tr>
<tr>
<td>PC45</td>
<td>61.0 ± 1.9 a</td>
<td>40.0 ± 1.4 a</td>
<td>19.0 ± 1.2 a</td>
</tr>
<tr>
<td>PC12</td>
<td>83.1 ± 1.4 a</td>
<td>32.0 ± 1.6 a</td>
<td>11.0 ± 0.8 a</td>
</tr>
<tr>
<td>PC11</td>
<td>70.0 ± 1.7 a</td>
<td>47.0 ± 2.0 a</td>
<td>33.0 ± 2.6 a</td>
</tr>
</tbody>
</table>

Table 1 Data scored after 6 weeks and represents the mean ±SE of at least 3 different experiments. In each column the values with different letters are significantly different (P<0.05) according to DMRT (Duncan’s multiple range test). Control = MSG medium without 24-epibrassinolide.

Table 2 Data scored after 14 weeks and represent the means (±SE) of at least 3 different experiments. Each values followed by the same letter in each column were not significantly different at P<0.5 according to DMRT (Duncan’s multiple range test).

*% of somatic embryogenesis = 5 g of embryogenic tissue of at least 3 different genotypes was taken aseptically and chopped into 100 pieces and subcultured on the maintenance medium for the growth of callus. Out of 100 pieces, growth of the number of pieces was recorded and calculated in terms of percentage of SE.

epiBr at 0.5, 1.0 and 2.0 μM (Table 1). Mature zygotic embryos produced the highest percentage of embryogenic tissue on half-strength MSG medium supplemented with 9.0 μM 2, 4-D and 2.0 μM 24-epiBr (initiation medium) in all five (PC05, PC45, PC173, PC12, and PC11) genotypes tested (Fig. 1A; Table 1). However, the percentage of somatic embryogenesis was not similar in these genotypes (Tables 1, 2). Therefore, the potential for somatic embryogenesis is first of all determined at the level of the genotype. The highest percentage of somatic embryogenesis (87.0 ± 1.8) was recorded in PC 05. On the other hand, the lowest percentage of somatic embryogenesis (61.0 ± 1.9) was obtained in PC45. In PC11, 70.0 ± 1.7 somatic embryos was noted whereas PC12 and PC173 recorded 83.1 ± 1.4 and 75.0 ± 2.0, respectively (Table 2).

The white mucilaginous embryogenic tissue was subcultured onto maintenance medium for the further development of ESMs (Fig. 1B). The pro-embryos that developed on the maintenance medium could not develop further until they were transferred onto medium with maltose, ABA and Gellan gum. The somatic embryos developed on maturation medium after 12-14 weeks in all 5 genotypes tested (Fig. 1C; Table 2). The total number of somatic embryos recovered/g FW of embryogenic tissue and somatic seedlings is listed in Table 2. After maturation, the advanced cotyledonary somatic embryos were selected for germination (Fig. 1D). Half-strength MSG medium without plant growth regulators was used as a germination medium. After 6 weeks, somatic seedlings were recovered (Fig. 1D). They were hardened in the greenhouse and ready for field transfer. On the basis of this study we confirmed that embryogenic competence is expressed finally at the level of single cells. Embryogenically competent cells are those which are capable of differentiating into embryos if they receive differentiation inducers (Malabadi et al. 2009a). The signals inducing competence and triggering embryogenic development are not easy to separate. In spite of the continuously increasing group of conifer species where the conditions for somatic embryo induction have been established, there are a number of species still recalcitrant to form somatic embryos. Highly embryogenic and recalcitrant genotypes exist even within a given species. It has been emphasized, however, that in many instances, recalcitrance could be resolved by optimizing growth conditions of plants or by proper explant selection (Malabadi and Nataraja 2003; Malabadi and van Staden 2005b; Malabadi and Nataraja 2006, 2007c, 2007d; Aronen et al. 2008; Malabadi et al. 2008a, 2008b, 2008c, 2008d, 2008e, 2009a, 2009c; Park et al. 2009).

24-epiBr and other brassinolides are ubiquitous in plants and elicit a wide spectrum of physiological responses (Grove et al. 1979; Yopp et al. 1981; Mandava 1988; Sakurai and Fujioka 1993; Mayumi and ShibaoKa 1995; Sasse...
formed buds. A maximum number of shoot buds per re-
curred at 0.1 or 1 μM 24-epiBr in which 44% of explants
(Sasaki 2002). The highest percentage of regeneration oc-
ted that brassinolide, a plant steroid lactone and the most
active brassinosteroid (BR), and its analogues enhanced
matured and increased crop yield of several vegetables,
cluding pepper. Since then, brassinolide has been regar-
ded as a new plant growth regulator which is essential for
ormal plant growth and development (Franck-Duchenne et al.
1998).

In angiosperms species, brassinosteroids have been
shown to have several effects, including stimulating cell
division, ethylene production, and adventitious tissue for-
mation and increasing resistance to abiotic stress (Brosa
1999). Although, little information is available for conifers,
 brassinosteroids have been isolated from conifers (Kim et al.
1990) and exogenous applications of brassinosteroids to
pine seedlings and spruce cuttings have shown improved root
growth. In vitro regenerating orchid plants (Renward et al.
reported that the use of brassinolide at 0.1 μM improved the
percentage of embryogenic cultures in loblolly pine, Doug-
las-fir (Pseudotsuga menziesii), and Norway spruce (Picea
abies). They have also showed that brassinolide increased
the weight of loblolly pine embryogenic tissue by 66% and
stimulated initiation in the more recalcitrant families of
loblolly pine and Douglas-fir, thus compensating somewhat
for genotypic differences in initiation (Pullman et al. 2003).
Embryogenic callus induction and growth of coffee and
potato was improved by the use of spirostane analogues of
BRs in the culture medium as a cytokinin substitute or com-
plement (Garcia 2000; More et al. 2004). Two spirostane
analogues of BRs (BB6 and MH5) were tested for callus
induction and plant regeneration in lettuce. Both BB6 and
MH5 enhanced callus formation and shoot regeneration from
lettuce cotyledons (Nunez et al. 2004). 24-epiBr at 2.0
μM with 9.0 μM 2,4-D enhanced the formation of em-
byogenic tissue from mature zygotic embryos on half-
strength MSG basal medium in Pinus wallachiana (Mala-
badi and Nataraja 2007a). Embryogenic callus induction
and growth of coffee, lettuce and potato was improved by the
use of spirostane analogues of BRs in the culture medium as a
cytokinin substitute or complement (Nakajima et al. 1992;
Clouse 1996; El-Bahnasawy et al. 2003). Successful initiation
of embryogenic tissue in cotton (Gossypium hirsutum),
organogenesis in sweet pepper (Capsicum annuum L. cvs.
‘Jupiter’ and ‘Pimiento Perfection’) and cauliflower (Brassica oleracea var. botrytis
L.) was established using 24-epiBr (Wang et al. 1992; Franck-Duchenne et al. 1998; Sasaki 2002). When hypocotyl
segments of cauliflower (Brassica oleracea var. botrytis
L.) were cultured on MS medium containing 0.1 μM
24-epiBr in the light, a significant stimulation of adven-
titious shoot regeneration was observed (Sasaki 2002).
Cytokinins (zeatin and iso-pentenylinamopurine) also
promoted shoot regeneration in B. oleracea (Sasaki 2002).
When 0.1 or 1 μM 24-epiBr was added together with these
cytokinins, maximum regeneration was further improved
(Sasaki 2002). Regeneration was much lower in the dark
due to increase ethylene synthesis in the 0.1 or 1 μM
2002). It was also noticed that when hypocotyl segments
of cauliflower were cultured in the light on MS medium con-
taining 24-epiBr at various concentrations, 0.1-10 μM 24-
epiBr significantly promoted adventitious bud formation
(Sasaki 2002). The highest percentage of regeneration oc-
curred at 0.1 or 1 μM 24-epiBr in which 44% of explants
formed buds. A maximum number of shoot buds per re-
generating explant was achieved at 1 μM 24-epiBr. Sasaki
(2002) also mentioned that zeatin also stimulated bud for-
mation in Cauliflower. But when 24-epiBr was added with
zeatin, regeneration was improved (91.7 vs. 42.3%) (Sasaki
2002). The interaction between cytokinin and BR suggests
that BR makes more cells competent to respond to the or-
ganogenic signal of the cytokinin and that these cells
became more sensitive to cytokinin (i.e., they required less
cytokinins to achieve a response (Sasaki 2002).

Successful initiation of PLBs and in vitro regeneration
was achieved using shoot tips (from mother plants grown
under greenhouse conditions) sections of C. elegans
and 24-epiBr-supplemented Mitra et al. (1976) basal
medium (Malabadi and Nataraja 2007b). The highest per-
centage of explants (91.0%) producing PLBs (24.0 ±
2.1) was recorded on 4.0 μM 24-epiBr and these PLBs or pro-
liferating shoot buds formed the maximum number of heal-
thed shoots (17.0 ± 1.23). Lower (0.5-1.0 μM) or higher
(6.0-20.0 μM) concentrations of 24-epiBr resulted in
the browning of explants and failed to produce PLBs. Initia-
tion of PLBs or proliferation of shoot buds decreased with an
increase in the concentration of 24-epiBr from 3.0 to 5.0
μM. All the newly formed PLBs survived and after nearly
12 weeks, small bud-like structures formed healthy shoots,
which rooted when cultured on basal medium supplemented
with 2.0 μM TRIA (Malabadi and Nataraja 2007b). Seedling
growth of rice plants was improved by 3 μM 24-epiBr treat-
ments (Ozdemir et al. 2004). Seedling growth of rice plants
was improved by 3 μM 24-epiBr treatment under salt stress
conditions. When seedlings treated with 3 μM 24-epiBr
were subjected to 120 mM NaCl stress, the activities of
superoxide dismutase, catalase and glutathione reductase
did not show significant difference, whereas the activity of
ascorbate peroxidase significantly increased (Ozdemir et al.
2004). In vitro regeneration of sweet pepper (Capsicum
annuum L. cv. ‘Jupiter’ and ‘Pimiento Perfection’) was
performed via direct organogenesis (Franck-Duchenne et al.
1998). The resulting shoot-buds of these two cultivars were
studied to see the influence of 24-epiBr on shoot bud
formation on media containing 0.1 μM 24-epiBr in the
presence or absence of 9.1 μM zeatin plus 5.2 μM gibberellic
acid for further stem elongation. Different responses to
these treatments were recorded depending upon the proto-
cols used and the genotypes tested. It appears that 24-epiBr
does not always act directly on stem elongation but may be
an elicitor and/or an enhancer of elongation in concert with
endogenous and other exogenously added PGRs in sweet
pepper. In vitro browning of 24-epiBr-treated explants was
more active than, or synergistic with, auxins such as IAA or
NAA (Brosa 1999). Oh and Clouse (1998) demonstrated
that brassinolide increased the rate of cell division in iso-
lated leaf protoplasts of Petunia hybridra. Hu et al. (2000)
suggested that 24-epiBr may promote cell division through
Cyc D3, a D-type plant cyclin gene through which cytoki-
nin activates cell division. In the same study, they also
showed that 24-epiBr can substitute cytokinin in culturing
Arabidopsis callus and suspension cells. Work with Chinese
cabbage protoplasts showed that 24-epiBR promoted cell division in the presence of 2,4-D and kinetin (Nakajima et al. 1996). However, few reports are available with respect to the effect of brassinolide in micropropagation and tissue culture.

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