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Role for Cytokinins in Somatic Embryogenesis of Pepper (*Capsicum annuum* L.)?

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Abstract The effects of cytokinins on somatic embryogenesis in pepper were studied using a Murashige and Skoog-based medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid and 10% sucrose. Compared to the medium without cytokinins, N^6-(Δ^2-isopentenyl)-adenine and kinetin had no significant effect, whereas benzylaminopurine and zeatin significantly reduced somatic embryogenesis. Coconut water (10%) either had no significant effect or it reduced embryogenesis as well. Induction of somatic embryogenesis was also dependent on genotype. Appearance of somatic embryos continued after removal of the embryos that developed first, especially on cultivars with a lower initial induction rate. Although somatic embryos germinated, the apical meristem did not elongate.

Keywords Apical meristem · Cytokinin · Coconut water · Embryogenesis · Hormone · Pepper · Somatic embryo

Introduction

Somatic embryogenesis (SE) in pepper could be a powerful tool to accelerate breeding and an alternative to seed production, especially in cases of incompatible crosses and poor seed setting. Despite its potential, few reports on this topic have been published (Harini and Sita 1993; Binzel and others 1996; Jo and others 1996; Bodhipadma and Leung 2002; Steinitz and others 2003) and regeneration of pepper via SE is not yet efficient enough and consistently reproducible to be described as a "protocol" or "system."

The developmental stages of plant regeneration via SE consist of three major steps: induction of SE, embryo development/maturation, and germination and growth of embryos. The third step has been the most important obstacle until now. Determination of organs in somatic embryos occurs at the early stages of development (Komamine and others 1992). Hence, although induction of somatic embryos has been achieved with varying success rates in previous studies, manipulation of the factors influencing induction of SE, the exogenous supplied plant growth regulators (PGR) in particular, could also successfully address the problem of plant development.

The role of auxins in the induction of SE has been extensively investigated (Steinitz and others 2003). However, because elevated auxin concentrations were used due to the addition of activated charcoal in the culture media, the optimum concentration levels are not known. Steinitz and others (2003) also suggested an inhibitory role for auxins in apical meristem development of pepper somatic embryos.

According to the concept of hormonal efficacy in SE, as outlined by Komamine and others (1992), auxin is necessary for initiation of embryogenesis, in particular for dedifferentiation of somatic cells, but the subsequent presence of auxin inhibits the development of somatic embryos.

Although their role in the dedifferentiation process is not clear, cytokinins are thought to be more involved in the differentiation and further development of morphogenetic

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events (Skoog and Miller 1957). Binzel and others (1996) reported that 10 μM thidiazuron (TDZ) was less efficient than coconut water (cw) in the induction of SE in pepper (Binzel and others 1996). Coconut water contains natural cytokinins such as zeatin (Z) and related forms, kinetin (KIN), and N^6-(Δ^2-isopentenyl)-adenine (iP)-like precursors (Ge and others 2006). In some other studies cytokinin supplementation was not always necessary for induction of SE (Jo and others 1996; Steinitz and others 2003). Thus, the role of cytokinins in the induction of SE in pepper is unclear; different cytokinins produce different responses and the optimum concentrations could be rather low. Also, immature zygotic embryos might have sufficient levels of endogenous cytokinins for induction of SE.

In this study we compared the effects of four different cytokinins on SE in pepper. In addition, SE in three different genotypes was studied, both from immature zygotic embryos and after the removal of the first somatic embryos.

Materials and Methods

Plant Material and Culture Conditions

Pepper plants of cvs Blue Star (Know-You Seed Co. Ltd, Taiwan) and Bendigo and Bell Boy (B & T World Seeds Ltd, France) were grown in a glasshouse at 18–20°C during the night and at 25–27°C during the day [16 h daylight supplemented by 400-W high-pressure sodium (SON/T) lamps].

Fruits harvested at the stage of mature green (Fig. 1A) were surface sterilized in 10% Domestos (5% NaClO) (Unilever, UK) for 20 min and then rinsed three times for 5 min with sterilized deionized water. Immature embryos, 5–6 mm long, were excised intact from immature seeds and placed in Petri dishes (5.5 cm diameter) containing 15 ml of culture medium (Fig. 1B). The endosperm of the immature seeds was in the liquid state, and when slight pressure was applied with the scalpel on the seed, both endosperm and embryo were expelled.

The basic medium was composed of MS salts and vitamins (Murashige and Skoog 1962), 2 mg/L 2,4-D, 10% cw, 10% sucrose, and 0.2% gelrite (Sigma, UK). According to Harini and Sita (1993), this medium produced the highest response and hereafter it is called HS medium. All media were adjusted to a pH of 5.6–5.8. All cultures were incubated at 25°C and 16 h light per day, at 40 µmol m^{-2} s^{-1} provided by Phillips 60–80-W cool-white fluorescent lamps. Results were recorded after 6-8 weeks.

Effect of Genotype and Coconut Water

A 3 × 2 factorial design (cultivars × media) was employed. Seventy immature embryos per cultivar were cultured on HS and HS medium without cw (HS-cw).

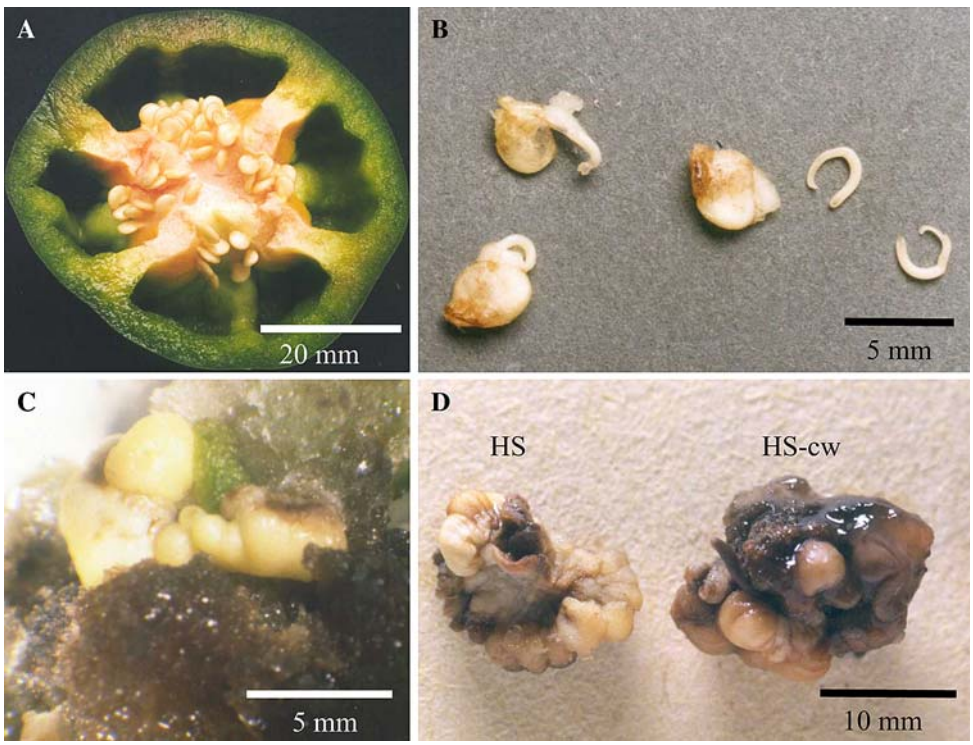

Fig. 1 **A** Horizontal section of pepper fruit cv Blue Star containing immature seeds. **B** Excision of embryos from immature seeds of pepper cv Blue Star. **C** Globular and heart-shaped somatic embryos of pepper cv Blue Star. **D** Somatic embryos of cv Blue Star on HS, HS-cw

Effect of Cytokinins

In a previous experiment cv Blue Star exhibited the highest embryogenic response (EmR) of the three cultivars and it responded in the same way on either HS or HS-cw media. Therefore, it was selected to test the effects of cytokinins on SE. Ten media treatments were constructed in a single-factor experimental design: HS and HS-cw media plus another eight HS-based media where cw was replaced by BAP, iP, KIN, or Z at 0.01 or 0.1 mg/L. Fifty-six immature zygotic embryos where cultured in each medium.

Second-Wave SE

Immature zygotic embryos were incubated for 8 weeks in HS medium and the somatic embryos were excised. After the removal of the somatic embryos, explants (30 per cultivar) were transferred to fresh HS medium and reincubated for 6 weeks.

Germination of Somatic Embryos

Somatic embryos of cv Blue Star were produced in HS medium. After excision, 20 and 28 somatic embryos, respectively, were placed on media containing MS salts and vitamins, 2% sucrose, 0.2% gelrite, and 0 or 1 mg/L gibberellic acid (GA_3).

Statistical Analysis

Data of the percentage of explants producing somatic embryos (EmR) were analyzed using the χ^2 test in Microsoft® Excel 97 SR-1. Data on the production of somatic embryos per responded explant (pSE) were analyzed using the F test and subsequently were subjected to Duncan's multiple-range test in SPSS® for Windows release 8.0. The level of significance in all comparisons was $p < 0.05$.

Results

Effect of Genotype and Coconut Water

Somatic embryos were first visible after 2 weeks, primarily arising from the apical meristem and cotyledon margins and less frequently from the hypocotyl. Their development continued for 3–4 weeks and was asynchronous (Fig. 1C, D). Torpedo-stage embryos had clearly distinguishable and separated cotyledons and were 6–8 mm long. Absence of cw did not affect EmR, but pSE was significantly higher on HS-cw than on HS medium. Orange-green to orange callus was produced in both media.

Genotype had significant but opposite effects on EmR and pSE. Cultivar Blue Star produced the highest EmR followed by cvs Bell Boy and Bendigo. Yield of somatic embryos (pSE) was almost two times higher for cv Bendigo than for cv Bell Boy (Table 1). Genotype-media interaction significantly affected EmR but not pSE. Cultivar Bell Boy responded better in HS-cw than in HS medium (Fig. 2).

Effect of Cytokinins

Induction and development features of somatic embryos were the same as in the previous experiment. Explants on media with 0% cw, 0.01 and 0.1 mg/L iP, and 0.01 mg/L KIN exhibited the highest EmR. Differences between these treatments were not significant (Table 2). EmR on 0% cw treatment was significantly higher than on 0.1 mg/L KIN, 0.01 mg/L BAP, 10% cw, 0.1 mg/L BAP, 0.01 mg/L Z, and 0.1 mg/L Z. EmR values of the two concentrations of each cytokinin were very close and exhibited a decreasing gradient, i.e. iP > KIN > BAP > Z; however, not all differences were significant. The highest pSE was produced

Table 1 Main effects on embryogenic response (EmR) and production of somatic embryos per responded explant (pSE) of two experimental factors, cultivars and media

	Cultivars			Media	
	Bendigo	Bell Boy	Blue Star	HS	HS-cw
EmR (%)	7.8[a]	65.0[b]	84.7[c]	54.6[a]	54.35[a]
pSE	10.0[a]	5.3[b]	7.6[ab]	3.0[a]	4.5[b]

Treatments with at least one letter the same are not significantly different

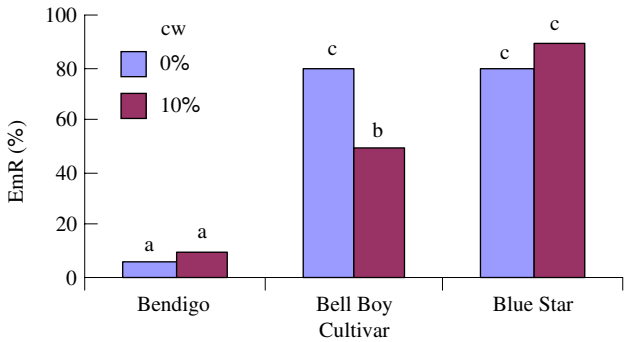

Fig. 2 Interaction of two experimental factors, i.e. cultivars and media, on embryogenic response (EmR). Treatments with at least one letter the same are not significantly different

Table 2 Embryogenic response (EmR) and production of somatic embryos per responded explant (pSE) of cv blue star on media containing cw and various cytokinins (single factor experiment)

Cytokinins	cw		iP		BAP		KIN		Z	
C (mg/L)	0%	10%	0.01	0.1	0.01	0.1	0.01	0.1	0.01	0.1
EmR (%)	75.0^{a}	42.5^{cd}	63.5^{abc}	63.3^{ab}	50.0^{bc}	30.8^{d}	57.1^{abc}	51.9^{bc}	29.3^{d}	6.2^{e}
pSE	4.7^{bcd}	2.5^{a}	4.2^{abcd}	5.1^{cd}	4.9^{cd}	2.8^{ab}	4.4^{bcd}	5.4^{d}	2.5^{a}	3.3^{abc}

Treatments with at least one letter the same are not significantly different

Table 3 Embryogenic response (EmR) and production of somatic embryos per responded explant (pSE) of three pepper cultivars during a second wave of induction of somatic embryos (single-factor experiment)

Cultivars	Bendigo	Bell Boy	Blue Star
EmR (%)	40.0^{a}	6.7^{b}	0.0^{b}
pSE	8.9^{b}	12.0^{a}	0.0^{c}

Treatments with at least one letter the same are not significantly different

on 0.1 mg/L KIN treatment, and it was significantly higher than on treatments with 0.01 mg/L Z, 10% cw, 0.1 mg/L BAP, and 0.1 mg/L Z (Table 2).

Second-Wave SE

Cultivar Bendigo exhibited the highest EmR and cv Bell Boy the highest pSE. Although cv Blue Star was the best-performing cultivar on the first-wave production of somatic embryos, it did not develop any somatic embryos on the second-wave production (Table 3).

Germination of Somatic Embryos

Germination of somatic embryos was 14.2% and 15% on media containing 0 and 1 mg/L GA_3, respectively (no significant difference). Although their roots elongated, the apical meristem did not elongate and remained in the same state for 8 weeks after transfer to germination media. A small amount of callus was also produced.

Discussion

The time to visible appearance of somatic embryos (2 weeks) was the same as previously reported (Harini and Sita 1993; Binzel and others 1996; Bodhipadma and Leung 2003), although Jo and others (1996) and Steinitz and others (2003) reported that somatic embryos were visible after 3 weeks. The first sign of SE induction was subepidermal cell divisions on cotyledons after incubation of 3-5 days (Bodhipadma and Leung 2003).

Binzel and others (1996) and Jo and others (1996) mentioned increased frequency of abnormal and fused embryos and suggested that it was due to a high number of cells becoming embryogenic in a limited area (Binzel and others 1996). We found that explants continued to induce somatic embryos after the initially induced somatic embryos were removed. This phenomenon was more apparent in explants of cultivars with low EmR during the first wave of somatic embryo production, suggesting either some kind of competition or dominance on endogenous hormone dynamics between developing embryos.

The apical meristem of somatic embryos failed to elongate during germination, making somatic embryos unable to resume normal vegetative development. Steinitz and others (2003) reported the same problem on media supplemented with high concentrations of 2,4-D and centrophenoxine (0.4–0.5 mM and 1.15–1.30 mM, respectively); these levels were used to counteract absorption of PGRs by activated charcoal added to the media. Plant production was improved when explants were transferred on PGR-free medium for 2 weeks after culture on induction medium (Bodhipadma and Leung 2002). Both earlier (Binzel and others 1996) and more recent (Bodhipadma and Leung 2003) histologic observations suggest that a shorter exposure to 2,4-D would be beneficial. On the other hand, in a separate experiment we found that no SE was induced in immature embryos cultured in modified liquid HS medium containing 1 mg/L 2,4-D for 1 week and then transferred on PGR-free solid medium. Instead, the embryos germinated and produced normal seedlings (unpublished data). These results show that auxins are necessary for the induction of SE and exposure time and concentration can be critical for apical meristem elongation.

Genotype-dependent differences in *in vitro* regeneration of pepper have been widely reported in both organogenesis (Ochoa-Alejo and Ramirez-Malagon 2001) and SE (Steinitz and others 2003). The present study concurred with the previous reports. Cultivars Bendigo and Blue Star responded in a similar way in the presence or absence of cytokinins (in the form of coconut water) in the culture media, whereas cv Bell Boy responded better in cytokinin-free media.

Factors directly affecting endogenous availability of PGRs are important sources of physiologic/morphogenetic variation. In pepper, genotype-dependent differences in the phenylopropanoid pathway have been shown (Holden and Yeoman 1994), and such differences may contribute to different responses to PGRs. In tobacco, coniferyl alcohol can be converted to dehydrodiconiferyl alcohol glucosides, which have cytokinin-like activity (Orr and Lynn 1992). Very little is known about the possible effects of dehydrodiconiferyl alcohol glucoside metabolism on the regulation of endogenous auxin and cytokinin levels.

Some studies attested to the necessity of cytokinins for the induction of SE (Binzel and others 1996), whereas others did not (Jo and others 1996; Steinitz and others 2003). When 10 μM (2.2 mg/L) TDZ was added in HS medium instead of or in addition to cw, EmR and pSE were lower than in HS medium (Binzel and others 1996). In the present study cytokinin supplementation could at best only match the EmR of cytokinin-free media. BAP and Z in particular significantly inhibited SE. The inhibitory effect on SE increased in the order iP < KIN < BAP < Z. Reasons for these activity differences are not known, but in some cases such differences (Mok and others 1978) are related to differential inactivation of cytokinins through cytokinin oxidase (Whitty and Hall 1974). Further research on cytokinin oxidase activity and its potential modulation by auxins (Coenen and Lomax 1997) and interaction with phenolic PGRs of the phenylopropanoid pathway could elucidate the mechanisms of embryogenesis in pepper and contribute to development of a method for clonal regeneration of this species.

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