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Factors Important for Somatic Embryogenesis in Zygotic Embryo Explants of *Capsicum annuum* L.

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We used four cultivars of *Capsicum annuum* L. -- Sweet Banana, California Wonder, Yolo Wonder, and Ace -- to re-examine the critical factors influencing somatic embryogenesis from zygotic embryo explants, as reported in the literature. When we followed the protocol of Buyukalaca and Mavituna (1996), which had induced somatic embryogenesis from mature zygotic embryos of cv. Ace, only callus was formed without embryogenesis from our mature zygotic embryo explants. Using the procedures of Harini and Lakshmi Sita (1993) and Binzel et al. (1996), with some modifications, we were able to induce somatic embryogenesis in all four cultivars. Rates of conversion were significantly reduced, from 75% and 65% to 40% and 28% in 'Sweet Banana' and 'California Wonder', respectively, when the immature zygotic embryo explants were held on the induction medium for longer than two weeks. Likewise, somatic embryogenesis of 'Yolo Wonder' was not observed if the induction medium was supplemented with 10% glucose or fructose, or without 10% sucrose. For somatic embryo induction and eventual plantlet conversion in 'Yolo Wonder', maltose could adequately replace sucrose. In all four cultivars, somatic embryos were initiated from immature zygotic explants on media with or without coconut water, under both light and dark conditions.

Keywords: coconut water, pepper, somatic embryogenesis, sugar

The fruits of domesticated *Capsicum* (pepper) cultivars are used in various forms as spices or vegetables, including differently colored dried powders, paprika, Tabasco, pungent chilli peppers, pimentos, red and cayenne peppers, and sweet or bell peppers. Although this produce is used primarily in the spice and pickling industry, whole-fruit consumption, especially of the mild pungent types, is increasing. Besides their popularity as foods and condiments, peppers are used in medicine and as ornamental plants (Purseglove et al., 1981; IBPGR, 1983; Langer and Hill, 1991).

Although experiments on in-vitro organogenesis of pepper are numerous (see Fári, 1986; Morrison et al., 1986; Ramage and Leung, 1996; Steinitz et al., 1999), little research has been reported for somatic embryo formation, e.g., via explant sources from immature zygotic embryos (Harini and Lakshmi Sita, 1993, Binzel et al., 1996, Jo et al., 1996); mature zygotic embryos (Buyukalaca and Mavituna, 1996); or the leaves of mature plants (Kintzios et al., 2000). The most attractive and convenient of these protocols is the one that uses mature embryos because one does not need to grow plants to the appropriate developmental stages in the glasshouse. However, it is unknown if this method is applicable to pepper cultivars other than 'Ace', for which it has been tested.

In this study we used four cultivars of *Capsicum annuum* L. 'Sweet Banana', 'California Wonder', 'Yolo Wonder', and 'Ace'. Our objective was to more closely examine some aspects of the published protocols using immature zygotic embryo explants for somatic embryogenesis in *Capsicum*.

MATERIALS AND METHODS

Plant Material

We obtained seeds of sweet pepper (*C. annuum* L.) cultivars Sweet Banana, California Wonder and Yolo Wonder 'B' from Arthur Yates & Co. Ltd., New Zealand. Those of cv. Ace came from Unwins Seeds Ltd., Histon, Cambridge, England.

Mature Zygotic Embryo Explants

We tested the protocol of Buyukalaca and Mavituna (1996) for all four cultivars. Mature dry seeds were immersed in distilled water overnight. After-

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Abbreviations: MS, Murashige and Skoog; 2,4-D, 2,4 dichlorophenoxy-acetic acid; GA_3, gibberellic acid; $AgNO_3$, silver nitrate.

ward, they were surface-sterilized by soaking them in 1% (v/v) sodium hypochlorite for 10 min and rinsing them three times with sterile distilled water. The seeds were then aseptically dissected to obtain embryo explants, which were placed on the following sequence of media as described by Buyukalaca and Mavituna (1996): 1) for embryogenic callus formation, mature zygotic embryos were cultured on an MS basal medium (Murashige and Skoog, 1962) supplemented with 9.05 μM 2,4-D and 3% (w/v) sucrose; (2) the embryogenic calli were subcultured in a liquid MS basal medium supplemented with 4.52 μM 2,4-D and 3% (w/v) sucrose; (3) the tissue was then pretreated in a liquid MS basal medium (KNO_3-free) supplemented with 6 g/L potassium citrate, 9.05 μM 2,4-D, and 3% (w/v) sucrose for three weeks; (4) embryo were initiated in a liquid MS basal medium supplemented with 6 g/L L-proline, 10 mM NH_4NO_3, and 3% (w/v) sucrose for three weeks; (5) embryos matured in the dark in a half-strength liquid MS basal medium supplemented with 1.89 μM ABA and 3% (w/v) sucrose; followed by (6) conversion into plantlets on a half-strength MS basal medium supplemented with 1.89 μM ABA and 3% (w/v) sucrose.

Immature Zygotic Embryo Explants

Plants of the four pepper cultivars were grown in potting mix supplemented with a slow-release (eight- to nine-month) fertilizer. They were grown separately in glasshouses at the University of Canterbury until flowers formed, fruit set, and seeds were produced. Each flower was labelled according to the day it opened. Green fruits were selected at various stages as our source of immature zygotic embryo explants used in somatic embryo induction, as described by Harini and Lakshmi Sita (1993) or Binzel et al. (1996). Our immature seeds were surface-sterilized by soaking in 1% (v/v) sodium hypochlorite for 7 minutes before being rinsed three times with sterile distilled water. Zygotic embryos were then isolated aseptically and placed on a somatic embryo induction and maturation medium comprising an MS basal medium supplemented with 2 mg/L 2,4-D, and 10% (w/v) each of coconut water (Sigma, St. Louis, USA) and sucrose (Harini and Lakshmi Sita, 1993). The resultant somatic embryos were transferred to an MS basal medium containing 2% (w/v) sucrose and 1 mg/L GA_3 for germination according to the protocol of Harini and Lakshmi Sita (1993). Here, we also tested the somatic embryo germination protocol of Binzel et al. (1996). That medium consisted of an MS basal medium supplemented with 2% sucrose, 10 μM $AgNO_3$ and 2.8 μM GA_3.

Media Modifications

The induction medium was essentially the same as that used by Harini and Lakshmi Sita (1993), except that it lacked the 10% (w/v) coconut water. Somatic embryo structures were transferred to a germination or conversion medium similar to that described by Binzel et al. (1996), except that we increased the concentration of $AgNO_3$ to 20 μM according to the protocol of Jo et al. (1996).

To study the effect on pepper somatic embryogenesis caused by different sugar types in the induction medium, we held immature zygotic embryos from 'Yolo Wonder' for two weeks on induction media that either had no added sugar, or had been supplemented with 10% (w/v) of glucose, fructose, maltose, or sucrose. Afterward, all the explants were transferred to the germination or conversion medium.

All media in this study were adjusted to pH 5.7, gelled with 0.8% (w/v) agar (Germantown Co., New Zealand), and autoclaved at 121 °C and 15 *psi* for 20 min. All the cultures were kept in a growth room at 22°C under continuous illumination from white fluorescent lamps, unless specified otherwise.

Statistical Analysis

Data were subjected to an analysis of variance. When required, means were compared using Tukey's test (HSD) from "Statistix for Windows version 7.0" (Analytical Software).

RESULT AND DISCUSSION

During the somatic embryo initiation step, calli developed well from mature embryos of 'Sweet Banana', 'California Wonder', and 'Yolo Wonder'. This demonstrates that these three cultivars responded in a similar manner as had 'Ace' on the callus induction medium described by Buyukalaca and Mavituna (1996). While 'Ace' showed nonembryogenic, white-watery calli, its yellowish-nodular-friable callus was highly embryogenic (Buyukalaca and Mavituna, 1996). Because 'California Wonder' and 'Yolo Wonder' had formed what appeared to be embryogenic callus, while 'Sweet Banana' had not (Table 1), only the calli initiated from the former two were investigated further.

Upon transfer to the liquid medium, cell suspensions from the calli of 'California Wonder' and 'Yolo

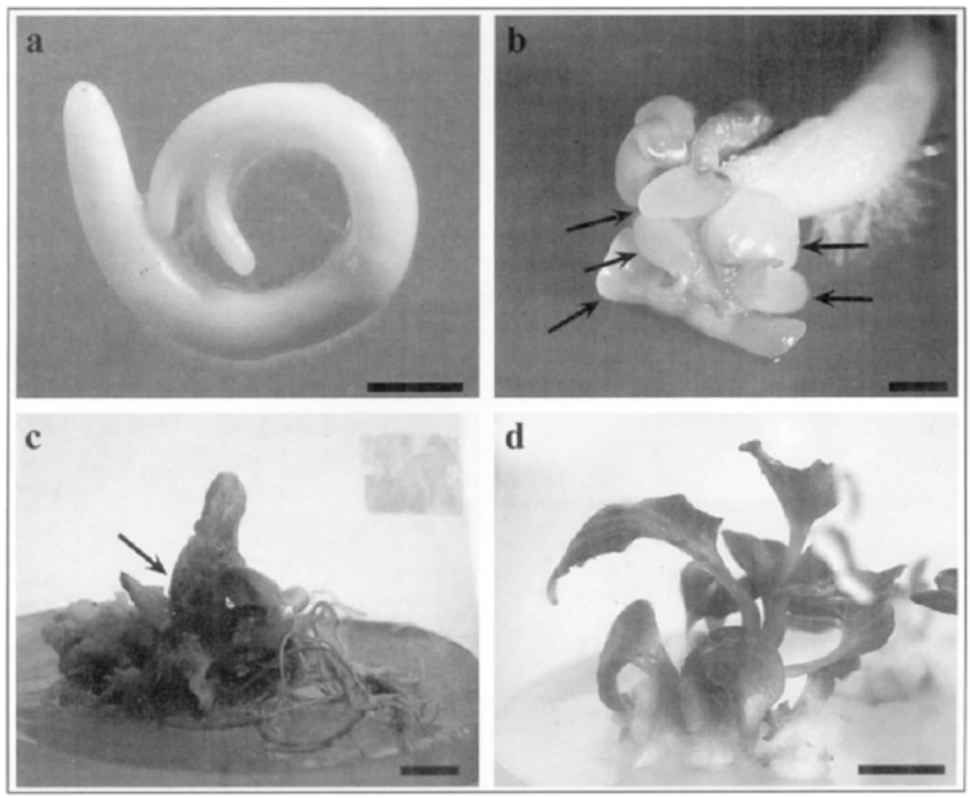

Figure 1. Somatic embryogenesis in *C. annuum* L. cv. Sweet Banana. **a,** typical immature zygotic embryo explant (scale bar = 1 mm) from glasshouse-grown plant; **b,** well-formed somatic embryo structures (arrows) on the embryonic axis and cotyledons of the immature zygotic embryo explant after three weeks of culture, scale bar = 1 mm; **c,** appearance of an abnormal plantlet (arrow), scale bar = 5 mm; **d,** appearance of a normal plantlet, scale bar = 5 mm.

Table 1. Appearance of calli formed from mature zygotic embryos of three cultivars of *C. annuum* L after four weeks of culture.

Cultivar	Callus morphology
Sweet Banana	White-watery
California Wonder	Yellowish-friable
Yolo Wonder	Yellowish-friable

Wonder' grew well. However, they failed to develop into somatic embryos after passing through the pretreatment and embryo initiation phases. This suggests that the success of this procedure is dependent on cultivar, and may work only with 'Ace'. However, even though we used mature embryos of 'Ace' as explants and faithfully following the original published procedure, no somatic embryos were formed. We have no explanation for this.

Immature Embryo as Explant Source

Immature embryos of 'Sweet Banana', 'Yolo Wonder', and 'Ace' developed somatic embryos on the embryo initiation and maturation medium described by Harini and Lakshmi Sita (1993). However, the percentage of structure formation was very low in all three cultivars (Fig. 1b). Likewise, very few plantlets resulted after the somatic embryo structures were transferred to the germination or conversion medium when we followed the protocol of Harini and Lakshmi Sita (1993). The main problem was that most of these structures developed friable or compact calli rather than converting to complete plantlets. In addition, most of those plantlets that did form showed abnormalities, including a lack of cotyledon-like or leaf-like structures, as well as twisting in the stems (compare Fig. 1, c and d).

Size of Immature Zygotic Embryos

Our preliminary study confirmed that size of the immature zygotic embryo was an important factor determining the success of somatic embryogenesis, as

suggested in previously published protocols. However, the impact of this factor varied among the cultivars. Immature zygotic embryos of five to seven millimeters showed the best response in all four cultivars. Other characteristics of the immature seed also seemed critical. For example, if the immature zygotic embryo appeared transparent, no somatic embryo structure was formed even if the embryo was of the appropriate size. In addition, the optimal embryo was not U-shaped, but rather had cotyledons that curled toward the embryonic axis (Fig. 1a). Likewise, those embryos isolated from seeds with fully-developed, cream-like endosperm that were still soft were more likely to form somatic embryos.

Time Held on Initiation and Maturation Medium

Following the procedures of Harini and Lakshmi Sita (1993) and Binzel et al. (1996), we held immature embryos in culture for 42 and 24 d, respectively, to obtain somatic embryos and eventual plantlet conversion. However, our preliminary experiment showed that if the immature zygotic embryos were left on that media for such a long period, somatic embryos were induced at a low rate, and abundant calli formed instead. In this research, the initiation and maturation media of Harini and Lakshmi Sita (1993) were modified by not adding any coconut water, and was termed "induction media".

If the immature zygotic embryos of 'Sweet Banana' and 'California Wonder' were left on the induction medium for only one week, no somatic embryo structures were formed. Instead, these embryos germinated and grew into normal seedlings. Unlike somatic embryogenesis in peanut, the optimum induction period was found to be seven days (Baker and Wetzstein, 1998).

Somatic embryo formation could be induced and tissue converted into plantlets after the embryos had been cultured on the induction medium for as little as two weeks before they were transferred to the germination or conversion medium (Table 2). In fact, somatic embryo structures were first observed after 12 to 14 d (sometimes 10 d) on the induction medium. The longer the immature embryos were left on this medium, the more visible these somatic embryo structures became. Moreover, the percentage of somatic embryo induction was not significantly different between the two- and four-week incubation periods. In contrast, the percentage of plantlet conversion notably decreased if explants with somatic embryo structures were held for more than two weeks on the induction medium. It is likely that during the first two weeks, 2,4-D may have stimulated formation of somatic embryo structures, but longer exposure may have inhibited subsequent development into complete plantlets.

Requirement for Coconut Water

One of the potential benefits of using coconut water in the media is that this source of cytokinin may enhance the induction of pepper somatic embryo when following the protocol of Harini and Lakshmi Sita (1993) and Binzel et al. (1996). However, the protocol of Jo et al. (1996) did not include this supplement in the induction medium when a different cultivar was tested. In the present research, somatic embryo structures from 'Sweet Banana', 'California Wonder', and 'Ace' formed equally well, if not better, on medium that lacked coconut water (Table 3). Likewise, the percentage of plantlet conversion did not differ significantly between the two coconut-water treatments (plus or minus) for 'Sweet Banana' and 'Ace', but it did differ for 'California Wonder'. Our test of 'Yolo Wonder' was conducted only on the medium without coconut water, and comparable high percentages of induction and conversion were obtained as with the other cultivars (data not shown).

Table 2. Influence of exposure duration (weeks) to somatic embryo-induction medium on somatic embryogenesis from immature zygotic embryo explants of *C. annuum* L. 'Sweet Banana' and 'California Wonder'.

Cultivar	weeks	Number of explants*	% Induction*	% Conversion*
Sweet Banana	2	82	78.813 ± 6.907a	74.994 ± 7.205a
	4	66	69.868 ± 11.755a	39.775 ± 10.068b
California Wonder	2	120	83.148 ± 6.028a	64.550 ± 8.403a
	4	89	86.837 ± 4.898a	28.265 ± 6.230b

*Values shown are means ± SE of data obtained from six and seven replicate experiments in each treatment for 'Sweet Banana' and 'California Wonder', respectively. Data from a cultivar followed by the same letter within a column are not significantly different (ANOVA, $P < 0.05$). For each treatment, number of explants = immature zygotic embryos used in all replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (with or without normal appearance).

Effect of Light and Dark Conditions

Light can be an important determinant of somatic embryo induction. For example, embryogenesis for the garden leek (*Allium porrum* L.) requires light (Hong and Debergh, 1995), whereas that process can occur in complete darkness for olive and Camellia (San-Jose and Vieitez, 1993; Rugini and Caricato,1995). In the red pepper (*C. annuum* L. cv. Nokkwang), somatic embryos were induced in the dark within three weeks (Jo et al., 1996), although the effect of light apparently was not studied specifically.

Immature zygotic embryo explants from 'Sweet Banana', 'California Wonder', and 'Yolo Wonder' were kept under continuous illumination or in the dark for about two weeks. Afterward, all the explants were transferred to the germination or conversion medium and kept under continuous illumination. Somatic embryo structures from all three were induced equally well in light or dark (Table 4). Moreover, plantlet conversion seems to be independent of prior induction under either light or dark conditions.

Table 3. Influence of somatic embryo-induction medium supplemented with or without coconut water on somatic embryogenesis from immature zygotic embryo explants of *C. annuum* L. 'Sweet Banana', 'California Wonder' and 'Ace'.

Cultivars	Coconut water	Number of explants*	% Induction*	% Conversion*
Sweet Banana	With	290	64.155 ± 3.985a	61.502 ± 3.798a
	Without	270	70.304 ± 3.974a	64.031 ± 4.236a
California Wonder	With	75	42.111 ± 10.594a	25.778 ± 8.588a
	Without	292	77.670 ± 4.160b	55.196 ± 5.724b
Ace	With	56	24.479 ± 4.687a	20.833 ± 4.886a
	Without	55	32.500 ± 5.114a	22.083 ± 9.087a

*Values shown are means ± SE of data obtained from 22 and 4 replicate experiments in each treatment for 'Sweet Banana' and 'Ace', respectively, and from 5 and 16 replicate experiments for 'California Wonder' with or without coconut water, respectively. Data from a cultivar followed by the same letter within a column are not significantly different (ANOVA, $P < 0.05$). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (with or without normal appearance).

Table 4. Effect of incubating immature zygotic embryo explants under continuous illumination or in the dark for two weeks on somatic embryogenesis of *C. annuum* L. 'Sweet Banana', 'California Wonder', and 'Yolo Wonder'.

Cultivar	Treatment	Number of explants*	% Induction*	% Conversion*
Sweet Banana	Light	61	54.762 ± 6.777a	49.048 ± 8.472a
	Dark	71	67.083 ± 9.373a	67.083 ± 9.373a
California Wonder	Light	71	68.095 ± 6.397a	54.976 ± 11.223a
	Dark	72	60.833 ± 4.439a	44.167 ± 7.377a
Yolo Wonder	Light	64	51.417 ± 6.939a	31.167 ± 8.691a
	Dark	66	51.742 ± 6.237a	36.818 ± 11.058a

*Values shown are means ± SE of data obtained from five replicate experiments in each treatment of the different cultivars. Data from a cultivar followed by the same letter within a column are not significantly different (ANOVA, $P < 0.05$). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (with or without normal appearance).

Table 5. Effect of 10% (w/v) maltose or sucrose in the somatic embryo induction medium on somatic embryogenesis from immature zygotic embryo explants of *C. annuum* L. cv. Yolo Wonder.

Treatment	Number of explants*	% Induction*	% Conversion*
Maltose	34	59.415 ± 11.210a	51.359 ± 13.832a
Sucrose	44	77.083 ± 6.468a	53.750 ± 15.251a

*Values shown are means ± SE of data obtained from four replicate experiments in each treatment. Data followed by the same letter within a column are not significantly different (ANOVA, $P < 0.05$). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (with or without normal appearance).

Effect of Sugar Type

Sugar, a carbon and energy source, can be a critical factor for somatic embryogenesis, although the type required varies by species. In *Panax ginseng*, the best regeneration of somatic embryos was obtained on a medium containing glucose (Tang, 2000) while somatic embryo production from *Hevea brasiliensis* was significantly higher on a maltose-containing medium (Blanc et al., 1999). In the current study, immature zygotic embryo explants of 'Yolo Wonder' were placed on induction media with or without different types of sugars for about two weeks. Afterward, they were transferred to the germination or conversion medium. Somatic embryo structures developed only on the media containing sucrose or maltose (Table 5). On the media supplemented without sugar or with glucose or fructose, the explants initially turned red and exhibited no further development (data not shown). The effect of sucrose versus maltose did not differ significantly (Table 5). However, no healthy plantlets were recovered after culture on the maltose-containing medium (data not shown). These results contradict those from other reports that demonstrated a promoter effect from maltose in a number of systems, e.g., asparagus somatic embryo development (Kunitake et al., 1997) and pollen embryogenesis in bell peppers (Dolcet-Sanjuan et al., 1997). Future studies should examine more closely the effect of maltose and other carbohydrates in somatic embryogenesis of different pepper cultivars.

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