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In vitro Flowering of Plantlets Regenerated via Somatic Embryogenesis from Immature Zygotic Embryo Explants of *Capsicum annuum* L. cv. Sweet Banana

By

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With 3 Figures

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Summary

BODHIPADMA K. & LEUNG D. W. M. 2002. In vitro flowering of plantlets regenerated via somatic embryogenesis from immature zygotic embryo explants of *Capsicum annuum* L. cv. Sweet Banana. – *Phyton* (Horn, Austria) 42 (1): 99–108, 3 figures. – English with German summary.

Plantlets of *Capsicum annuum* L. cv. Sweet Banana (*Solanaceae*) regenerated from excised immature zygotic embryos cultured on a modified protocol for somatic embryogenesis were capable of flowering in vitro. The in vitro flowers were compared with those produced by the mature plants grown in the glasshouse. On the day of flower opening, both in vitro and in vivo flowers were collected for the present study. It was found that there were no obvious differences in the outer parts (calyx colour, corolla lobe number, corolla colour and corolla length) of both sources of flowers. The inner parts of both in vitro and in vivo flowers were slightly different as far as stamen number, anther length, filament length, ovary length and style length are concerned. However, anther colour, filament colour, ovary number, ovary colour, style colour, and stigma colour were the same. Pollen from both in vitro and in vivo flowers also appeared to be the same when observed under a scanning electron microscope. In contrast, pollen physiology of in vitro and in vivo flowers in relation to both viability and germination tests were slightly different. The percentages of viability and germination of pollen from in vitro flowers were about 94% and 25%, respectively, while those of pollen from in vivo flowers were about 96% and 34%, respectively.

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Zusammenfassung

BODHIPADMA K. & LEUNG D. W. M. 2002. In vitro-Blüte von Pflanzen, die aus unreifen zygotischen Embryonen von *Capsicum annuum* L. cv. Sweet Banana über somatische Embryogenese regeneriert wurden. – *Phyton* (Horn, Austria) 42(1): 99–108, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Pflänzchen von *Capsicum annuum* L. cv. Sweet Banana (*Solanaceae*), die aus entnommenen, unreifen, zygotischen Embryonen regeneriert und nach einem modifizierten Rezept für somatische Embryogenese kultiviert worden waren, sind in der Lage, in vitro zu blühen. Die in vitro-Blüten wurden mit Blüten von ausgewachsenen Gewächshauspflanzen verglichen. In vitro- und in vivo-Blüten wurden am ersten Anthesetag untersucht. Es gibt keine augenfälligen Unterschiede in den äußeren Blütenteilen (Kelch-Farbe, Kronzipfel-zahl, Kronen-Farbe und Kron-Länge). Die inneren Teile von in vitro- und in vivo-Blüten zeigen leichte Unterschiede hinsichtlich Staubblatt-Zahl, Antheren-Länge, Filament-Länge, Ovarium-Länge und Griffel-Länge. Dagegen sind Antheren-Farbe, Filament-Farbe, Ovar-Zahl, Ovar-Farbe, Griffel- und Narben-Farbe ident. Die Pollenmorphologie, im Lichtmikroskop und im REM untersucht, ist ebenfalls ident, während es hinsichtlich Vollkörnigkeit und Keimung leichte Unterschiede gibt: Die Prozentsätze für lebende Pollenkörner bzw. Keimung liegen bei in vitro-Blüten bei 94% und 25%, bei in vivo-Blüten bei 96% und 34%.

1. Introduction

Capsicum annuum L. is an economically important species, being used as a food, spice and ornamental (RYLSKI 1986: 341–354). The cultivar 'Sweet Banana' has a sweet taste. It is also good for frying and decoration (HARTMANN & al. 1981: 542–544). There have been many in vitro experiments of pepper investigating both de novo organogenesis and somatic embryogenesis (e.g. MORRISON & al. 1986; HARINI & LAKSHMI SITA 1993; BINZEL & al. 1996; BUYUKALACA & MAVITUNA 1996; RAMAGE & LEUNG 1996; KINTZIOS & al. 2000). In addition, there is a report on preliminary observations showing that the plants regenerated from protoplast culture (SAXENA & al. 1981) or excised shoot tips from seedlings (TISSERAT & GALLETTA 1995) of *Capsicum frutescens* L. cv. California Wonder flowered in vitro.

In vitro flowering of many plant species has been achieved and applied to study the factors involved in the regulation of flowering (SCORZA 1982). Nonetheless, in vitro flowers might be smaller (NADGAUDA & al. 1997) and malformed (HARADA & MURAI 1998).

Here, we report, for the first time, in vitro flowering of the plantlets regenerated via somatic embryogenesis from immature zygotic embryos of *Capsicum annuum* L. cultivar Sweet Banana. Furthermore, we showed that the in vitro flowers were highly comparable to those produced by the mature plants grown in the glasshouse. As far we are aware, this is also the first study comparing in vitro and in vivo flowers of *Capsicum annuum* L.

2. Materials and Methods

Plant Material

Seeds of sweet pepper, *Capsicum annuum* L. cv. Sweet Banana were obtained from Arthur Yates & Co Ltd., Auckland, New Zealand. After germination, plants were grown in a potting mix with a slow releasing fertilizer lasting for 8–9 months supporting flower formation, fruit set and seed production in the glasshouse at the University of Canterbury. From the glasshouse-grown plants, immature embryos were obtained for somatic embryo induction as described previously with some modifications (HARINI & LAKSHMI SITA 1993; BINZEL & al. 1996). The immature embryos (5–7 mm) were excised and placed on the induction medium (see Media). The in vitro cultures were kept in a growth room at 22 °C under continuous illumination provided by white fluorescent lamps. Plants regenerated from the somatic embryos (BODHIPADMA & LEUNG unpublished observations) were maintained in the plantlet development medium (see Media) until flowers were developed. All the studies in this paper were carried out on flowers produced by the plants in the glasshouse and those by plantlets regenerated from the somatic embryos under in vitro conditions. In this paper, the former are referred to as 'in vitro' flowers and the latter as 'in vivo' flowers. All the flowers were harvested on the day of their opening.

Media

The induction medium was modified from HARINI & LAKSHMI Sita 1993 and BINZEL & al. 1996 using MS basal medium (MURASHIGE & SKOOG 1962) containing 2 mg/l 2,4-D with or without 10% coconut water and 10% (w/v) sucrose. Somatic embryonic structures were then transferred to a conversion or germination medium comprising MS basal medium supplemented with 1 mg/l GA₃, 2% sucrose and 20 µM AgNO₃. The plantlet development medium was MS basal medium containing 1 mg/l NAA. All media were adjusted to pH 5.7, gelled with 0.8% (w/v) agar (Germantown Company, New Zealand) and autoclaved at 121 °C and 15 psi for 20 minutes.

Flower Characters

At least 20 in vitro and 50 in vivo flowers from 16 and 25 plants, respectively, were examined immediately after harvesting. The following parameters were studied: flower shape, calyx colour, corolla lobe number, corolla colour, corolla length, stamen number, anther colour, anther length, filament colour, filament length, ovary number, ovary colour, ovary length, style colour, style length and stigma colour.

Pollen Morphology

Stamens of a flower (usually 5–6 stamens per flower) were excised and placed on a glass slide until the anthers dehisced and released most of their pollen grains. Then all the stamens were discarded and the pollen were mounted on stubs covered with carbon conductive tabs for coating without intermediate drying. The stubs were placed straight into a diode sputter coater (SEM coating unit E5000, Polaron equipment limited, England). All specimens were coated with gold paledium for 5 minutes at high tension of 1.2 kV at 50 mA to deposit 500 nm of gold. The stubs were removed from the coater and were examined immediately using a scanning electron microscope (Leica S440, Cambridge Instruments Ltd, Cambridge, England). SEM operation was manipulated using secondary electrons at 50 pA at 15 kV. After this both in vitro

and in vivo pollen grains were observed and the chosen images were recorded in the tif file format. Pollen size was measured from 6 different fields of observation and at least 60 pollen grains from at least 3 in vitro or in vivo flowers from different plants were examined.

Pollen Viability

Pollen grains (collected as described before) of at least six flowers from different plants were mixed with 2 drops of Alexander's stain (ALEXANDER 1969) on a glass slide, mounted with a cover slip, and warmed over a Bunsen burner's flame for a few seconds. After this the pollen grains were examined under a light microscope. The aborted pollen grains stained green while non-aborted ones red to deep red.

Pollen Germination

The medium of MERCADO & al. 1994 comprising 0.1 mM boric acid, 1 mM calcium chloride di-hydrate, 5% (w/v) sucrose, 0.8% (w/v) agar was used for both in vitro and in vivo pollen germination test. It was autoclaved at 121 °C and 15 psi for 20 minutes, before being dispensed into plastic Petri dishes (80 mm diameter). Pollen grains of at least six flowers from different plants were collected as described before and were dusted uniformly over the surface of the medium in an agar plate. All the Petri dishes were wrapped in polyethylene film and incubated at 26 °C in a dark room. Germination of pollen from both in vitro and in vivo flowers were examined under a light microscope over a period of 24 hours.

Statistical Analysis

Data were first subjected to analysis of variance (ANOVA, $P < 0.05$) to see if the means are different and if required, Tukey (HSD) comparison of means was performed by using "Statistix for Windows version 7.0" (Analytical Software).

Abbreviations: SEM, scanning electron microscope; MS, MURASHIGE & SKOOG; 2,4-D, 2,4 dichlorophenoxy acetic acid; GA₃, gibberellic acid; AgNO₃, silver nitrate; NAA, α naphthalene acetic acid

3. Results and Discussion

Flower Structures

In vitro and in vivo flowers of *Capsicum annuum* L. cv. Sweet Banana were harvested for this comparative study on the day of their opening. The shape of both types of flowers appeared to be the same (Figure 1). A closer examination confirmed that there were no obvious differences in the outer parts (calyx colour, corolla lobe number, corolla colour, and corolla length) of both in vitro and in vivo flowers (Table 1.1). However, a comparison of the inner parts of both types of flowers revealed that stamen number, anther length, filament length, ovary length and style length were slightly different (ANOVA, $P < 0.05$) whereas anther colour, filament colour, ovary number, ovary colour, style colour, and stigma colour were the same (Table 1.2). The in vitro flowers tended to have more stamens, longer filaments and style, but smaller anthers and shorter ovary than in vivo flow-



Fig. 1. Appearance of in vitro (on the left) and in vivo (on the right) flowers of *Capsicum annuum* L. cv. Sweet Banana. – Scale bar = 10 mm.

Table 1.
Comparison between in vitro and in vivo flower characters of *Capsicum annuum* L. cv. Sweet Banana

1.1 Outer parts^a

Source	Flower shape	Calyx colour	Corolla lobe number	Corolla colour	Corolla length (mm)
In vitro	Bell type	Green	5.6 ± 1338a	White	9.425 ± 0.2697a
In vivo	Bell type	Green	5.34 ± 0.0677a	White	9.38 ± 0.1074a

1.2 Inner parts^a

Source	Stamen number	Anther colour	Anther length (mm)	Filament colour	Filament length (mm)
In vitro	5.75 ± 0.1758a	Purple	2.6 ± 0.0712a	White	2.225 ± 0.0571a
In vivo	5.26 ± 0.0627b	Purple	3.125 ± 0.0344b	White	2.21 ± 0.0555b

Source	Ovary number	Ovary colour	Ovary length (mm)	Style colour	Style length (mm)	Stigma colour
In vitro	1	Green	6.275 ± 0.2653a	White	3.375 ± 0.2142a	Green
In vivo	1	Green	6.82 ± 0.0805b	White	2.78 ± 0.0771b	Green

^a Values are means of 20 replicates ± S.E. from in vitro flowers and 50 replicates ± S.E. from in vivo flowers on the day of their opening. Data marked by same letter in a column are not significantly different (ANOVA, *P* < 0.05).

ers. This may be caused by in vitro condition or somaclonal variation of the flowers. The significance of these small differences in the flower parts to reproductive success is not known.

Overall, the data indicate that the in vitro flower of *Capsicum annuum* L. cv. Sweet Banana were well-formed and comparable to the in vivo flowers in many visual features. Similar findings were found in several recent studies (e.g. CARSON & LEUNG 1994a, b; NADGAUDA & al. 1997; ZHANG & LEUNG 2000).

Pollen Morphology and Physiology

It has been suggested that pollen should be collected soon after anther dehiscence for morphological and physiological investigations (SHIVANNA & RANGASWAMY 1992: 5–7). In general, the glasshouse-grown *Capsicum annuum* L. cv. Sweet Banana plants released most of their pollen in the morning of flower opening. In contrast, the time of collecting pollen from the in vitro flowers was less predictable, presumably because the in vitro flowers were under 24 hours of continuous lighting in the growth room.

There are few publications on pollen of the *Solanaceae* (MURRY & ESHBAUGH 1971; PUNT & MONNA-BRANDS 1977; KUMAR & al. 1987), especially on pollen morphology of *Capsicum annuum* L. The present SEM observations revealed that the morphology of pollen from both in vitro and in vivo flowers is not different (Table 2, Figure 2). The grains are tricolporate as described e.g. in MOORE & al. 1991: 133–143.

Using Alexander's staining method (1969), it was found that over 90% of the pollen from both sources of flowers were viable. However, the pollen grains from in vitro flowers were marginally less viable than those from in vivo flowers (Table 2).

The in vitro germination test showed that the pollen of in vivo flowers performed better than those of in vitro flowers (Table 2).

BREWBAKER & KWACK 1963 reported that the percentage of germination in *Capsicum* varied from 2–35%. In this study with the cultivar 'Sweet Banana', about 34 and 25% of the pollen from in vivo and in vitro flowers germinated (Table 2), respectively, although MERCARDO & al. 1994 used the same medium and observed *Capsicum* pollen germination close to 50%. Pollen of in vitro flowers germinated slower than those of in vivo flowers. No germination of pollen from either source resulted after 15 hours from the start of the germination test (Figure 3). This was different from another study showing that no pollen of *Capsicum annuum* L. F1 hybrid 'Latino' germinated after 6 hours. This suggested that pollen germination may vary among pepper cultivars.

4. Acknowledgements

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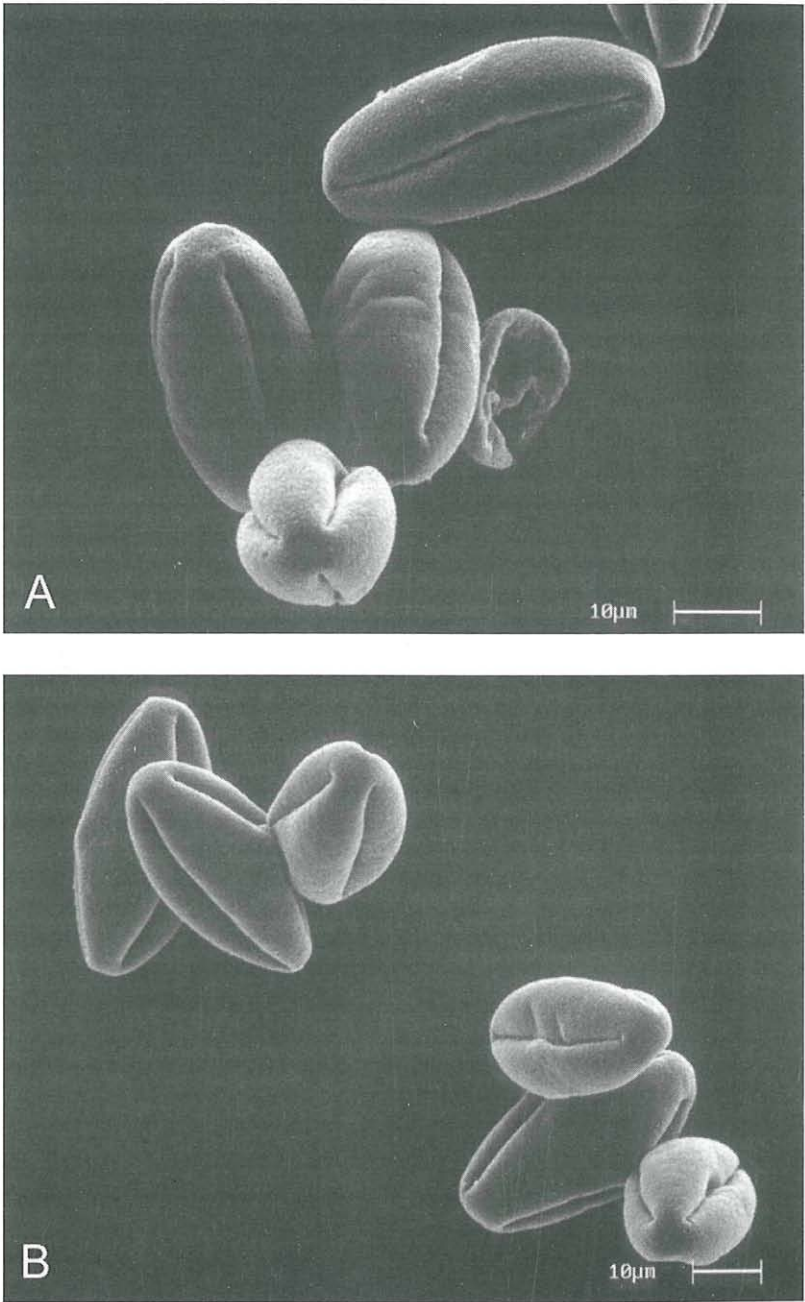


Fig. 2. SEM observations of pollen grains from in vitro (A) and in vivo (B) flowers of *Capsicum annuum* L. cv. Sweet Banana. – Scale bar = 10 mm.

Table 2.
Comparison of the morphology, viability and germination of pollen grains from in vitro and in vivo flower of *Capsicum annuum* L. cv. Sweet Bananaa

Source	Pollen type	Pollen morphology (from SEM)			Pollen physiology		
		Grain shape	Exine	Pollen length in	Pollen viability	Pollen germination ^b	
		Equatorial view	sculpture	equatorial view (µM)	(%)	(%)	
In vitro	Trizonocolporate	Elliptic	Scabrate	39.355 ± 0.2275 ^a	94.219 ± 0.8449 ^a	25.169 ± 1.3929 ^a	
In vivo	Trizonocolporate	Elliptic	Scabrate	39.892 ± 0.2095 ^a	96.271 ± 0.5425 ^b	34.408 ± 1.7587 ^b	

^a Values are means of 60 pollen grains ± S.E. from in vitro and in vivo flowers on the day of their opening except those of pollen viability and pollen germination in which pollen grains from 6 replicate flowers were used. Data marked by same letter in a column are not significantly different (ANOVA, $P < 0.05$).

^b Pollen form in vitro and in vivo flowers began to germinate 45 and 30 minutes, respectively, after the start of the germination test. Data were obtained after 24 hours later.

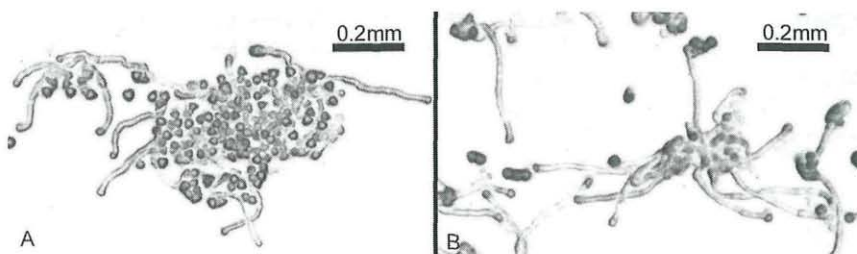


Fig. 3. Germination of pollen from in vitro (A) and in vivo (B) flowers of *Capsicum annuum* L. cv. Sweet Banana. The photo was taken 15 hours after the start of the in vitro germination test. – Scale bar = 0.2 mm.

and light microscopy. We also gratefully acknowledge the assistance of Supanya BODHIPADMA in the glasshouse. This work was supported in part by a doctoral scholarship to K. BODHIPADMA from the Ministry of University Affairs, Thailand.

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