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Histological Changes during Direct Somatic Embryogenesis from Immature Zygotic Embryo Explants of *Capsicum annuum* L. cv. Sweet Banana

By

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With 6 figures

Key words: *Capsicum annuum* L., histology, pepper, somatic embryogenesis.

Summary

BODHIPADMA K. & LEUNG D. W. M. 2003. Histological changes during direct somatic embryogenesis from immature zygotic embryo explants of *Capsicum annuum* L. cv. Sweet Banana. – *Phyton* (Horn, Austria) 43 (1): 197-206, 6 figures. – English with German summary.

Direct somatic embryogenesis was initiated from immature zygotic embryo explants of *Capsicum annuum* L. cv. Sweet Banana. Globular embryonic structures were easily observed under a stereo-microscope after 14 days of culture on induction medium containing 2,4-D. These structures turned green 5-7 days after explants were transferred to germination or conversion medium devoid of 2,4-D, and then they germinated into plantlets about 3 days later. The origin and developmental sequence of somatic embryos were investigated using a resin infiltration and embedding technique. Periclinal cell division firstly began underneath the protoderm of the cotyledons of explant after 3 days of culture on induction medium. The number of cells obviously increased after 5-7 days of culture as a result of both periclinal and anticlinal cell divisions. Upon further culture on germination or conversion medium, other well known somatic embryo developmental stages, including heart-shaped, torpedo and cotyledonary stages were observed.

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Zusammenfassung

BODHIPADMA K. & LEUNG D.W.M. 2003. Histologische Veränderungen während direkter somatischer Embryogenese von unreifem zygotischen embryonalen Pflanzenmaterial bei *Capsicum annuum* L. cv. Sweet Banana. – *Phyton* (Horn, Austria) 43 (1): 197 – 206, 6 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die direkte somatische Embryogenese wurde bei unreifem zygotischen embryonalen Pflanzenmaterial von *Capsicum annuum* L. cv. Sweet Banana eingeleitet. Nach 14-tägiger Kultur auf dem Induktionsmedium, welches 2,4 D enthielt, konnten die kugeligen embryonalen Strukturen sehr leicht unter einem Stereomikroskop beobachtet werden. Nach 5 bis 7 Tagen ergrüneten diese Strukturen auf einem 2,4 D-freien Keim- oder Konversionsmedium und 3 Tage später entwickelten sie sich zu Pflänzchen. Die Entstehung und der Ablauf der Entwicklung der somatischen Embryos wurden nach Harzinfiltration und Einbettung untersucht. Als erstes zeigten sich nach 3 Tagen Kultur auf dem Induktionsmedium perikline Zellteilungen unterhalb des Protoderms der Keimblätter des Ausgangsmaterials. Nach 5 bis 7 Tagen Kultur war die Anzahl an Zellen als Folge von sowohl periklinen als auch antiklinen Zellteilungen deutlich erhöht. Nach weiterer Kultur auf dem Keimungs- oder Konversionsmedium konnten auch alle anderen somatischen Entwicklungsstadien, wie herzförmige, torpedoförmige, und Keimblatt-Stadien beobachtet werden.

Introduction

Somatic embryogenesis in *Capsicum* (peppers) could be induced using immature zygotic embryo explants (HARINI & LAKSHMI SITA 1993, BINZEL & al. 1996, Jo & al. 1996, BODHIPADMA & LEUNG 2002), mature zygotic embryo explants (BUYUKALACA & MAVITUNA 1996) or the leaves of mature plants (KINTZIOS & al. 2000). However, only two of these reports showed limited histological evidence of somatic embryo formation (HARINI & LAKSHMI SITA 1993, BINZEL & al. 1996). In particular, the origin and sequence of pepper somatic embryogenesis are still unclear.

Unlike other previous studies (HARINI & LAKSHMI SITA 1993, BINZEL & al. 1996, JO & al. 1996), we found that culturing of immature *Capsicum* zygotic embryo explants on induction medium containing 2,4-D for a much shorter time, 12-14 days, resulted in little or no callus formation and subsequent transfer to germination or conversion medium devoid of 2,4-D plant regeneration occurred (BODHIPADMA & LEUNG 2002). Although it has been suggested (without the required histological evidence) that somatic embryo induction, development and maturation could be completed on induction medium containing 2,4-D (HARINI & LAKSHMI SITA 1993, BINZEL & al. 1996), it is questionable what, if any of the changes associated with somatic embryo development could occur in our somatic embryo induction protocol.

Here, both morphological and histological changes occurring during direct somatic embryogenesis (i.e. without accompanying callus formation) in immature zygotic embryo explants of *Capsicum annuum* L. cv. Sweet Banana are reported.

Abbreviations: 2,4-D, 2,4 dichlorophenoxy acetic acid; GA₃, gibberellic acid; MS, MURASHIGE & SKOOG 1962, AgNO₃, silver nitrate.

Material and Methods

Plant material

Seeds of sweet pepper, *Capsicum annuum* L. cv. Sweet Banana were obtained from Arthur Yates & Co Ltd., New Zealand. Plants of this cultivar were grown in a potting mix with a slow releasing fertiliser lasting for 8-9 months at the glasshouse of the University of Canterbury until flower formation, fruit set and seed production. Each flower was labelled on the day of flower opening. Selected green fruits were used to obtain immature zygotic embryo explants for somatic embryo induction.

Somatic embryo induction and culture

Immature pepper seeds from the selected fruits were surface sterilised by soaking in 1% (v/v) sodium hypochlorite for 7 minutes before they were rinsed three times with sterile distilled water. Zygotic embryo was isolated aseptically and was then placed on induction medium (MS basal medium supplemented with 2 mg/l 2,4-D and 10% (w/v) sucrose) which was essentially the same as that of HARINI & LAKSHMI SITA 1993 except without 10% (w/v) coconut water. Somatic embryo structures were then transferred to germination or conversion medium (MS basal medium containing 1 mg/l GA₃, 20 µM AgNO₃ and 2% (w/v) sucrose) which was the same as that of BINZEL & al. 1996 except the increased concentration of AgNO₃ used here.

All media in this study were adjusted to pH 5.7, gelled with 0.8% (w/v) agar (Germantown company, New Zealand) and autoclaved at 121°C and 340 kPa for 20 minutes. All cultures were kept in a growth room at 22 °C under continuous illumination (average 26.5 µE/sec/m², measured by 21X Micrologger, Campbell Scientific Inc., USA) provided by white fluorescent lamps.

Morphological examination

Immature zygotic embryo explants of *C. annuum* L. cv. Sweet Banana were taken out of tissue culture containers at day 0, 3, 5, 7, 10 and 14 after culture on induction medium and at day 3, 5, 7 and 10 after culture on germination or conversion medium. At least 3 explants were obtained in each of these sampling times for examination under a dissection microscope.

Histological Procedures

Zygotic embryo explants (referred to as 'samples' later) of *C. annuum* L. cv. Sweet Banana were taken out of the tissue culture containers at day 0, 3, 5, 7, 10, 14 and 16 after culture on induction medium and at day 3, 5, 7 and 10 after culture on germination or conversion medium. The samples were placed immediately into vials with glutaraldehyde fixative (3 % (v/v) glutaraldehyde in 0.075 M phosphate buffer at pH 7.2) and was kept overnight.

The samples were dehydrated in the vials with a graded ethanol series for at least one hour. Then they were transferred into 100% ethanol and was kept overnight. The absolute ethanol was replaced by infiltration with resin of hydroxyethyl-methacrylate (Kulzer and Co., GmbH, Wehrheim, Germany) and the infiltration solution was prepared from a Technovit® 7100 Resin kit. The manufacturer's instructions were followed for resin infiltration, embedding and polymerisation.

Sections were cut using a Reichert-Jung 2040 Autocut Rotary microtome (Cambridge Instruments GmbH, Germany) mounted with a Ralph type glass knife

made on an LKB 2078 HistoKnifemaker (LKB-Produkter AB, Sweden). The sections were stained with a mixture of aqueous 1% (w/v) methylene blue (Gurr®) and 1% (w/v) Azure II (Gurr®), and were examined under a light microscope.

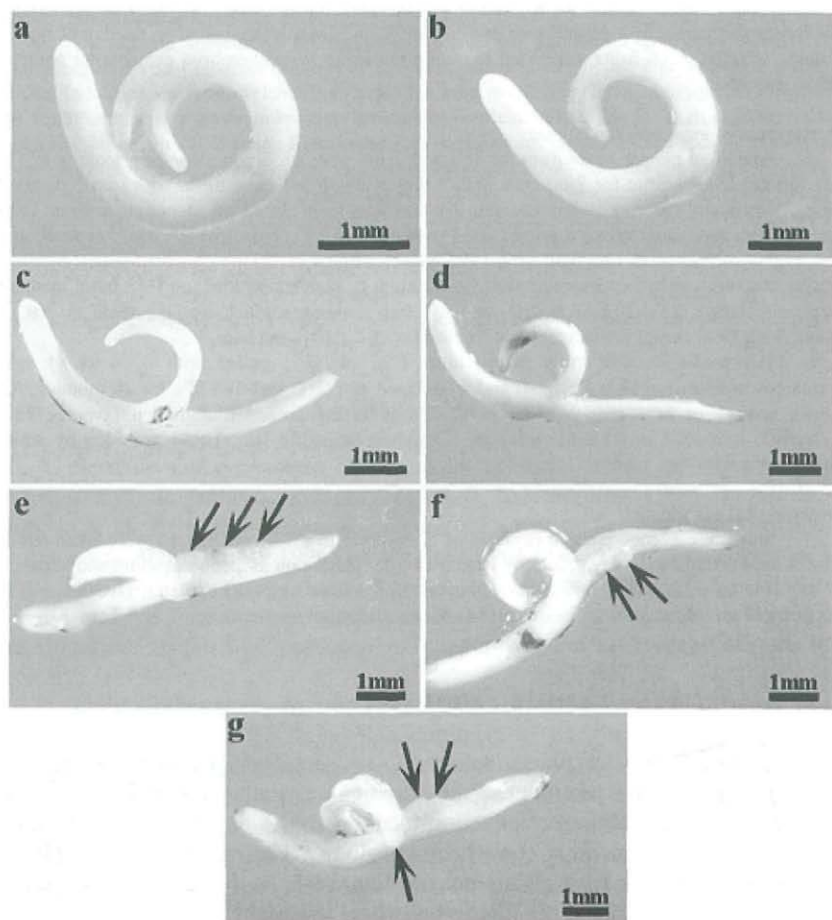


Fig. 1. Macroscopic changes on immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana placed on somatic embryo induction medium for different times. The arrows indicate structures putatively associated with somatic embryogenesis. a. day 0; b. day 3; c. day 5; d. day 7; e. day 10; f. day 14; g. day 16.

Results and Discussion

Morphology of somatic embryo formation

At an early stage of culture on induction medium (day 3), the cotyledons of immature zygotic embryo explants of *C. annuum* L. cv. Sweet Banana remained curling toward the embryonic axis as on day 0 (Figs. 1 a and b). After 5 days of culture, the cotyledons split further apart (Fig. 1 c). After this about 5-7 days on induction medium, somatic embryo structure was first barely visible (Fig. 1 d). Somatic embryo structures were more easily seen with the aid of a microscope after 14-16 days of culture on the induction medium (Figs. 1 f and g). Some of these macroscopic structures could be found as early as day 10 (Fig. 1 e). Similar observations were obtained during culture of immature zygotic embryo explants of cvs. California Wonder, Yolo Wonder and Ace (data not shown).

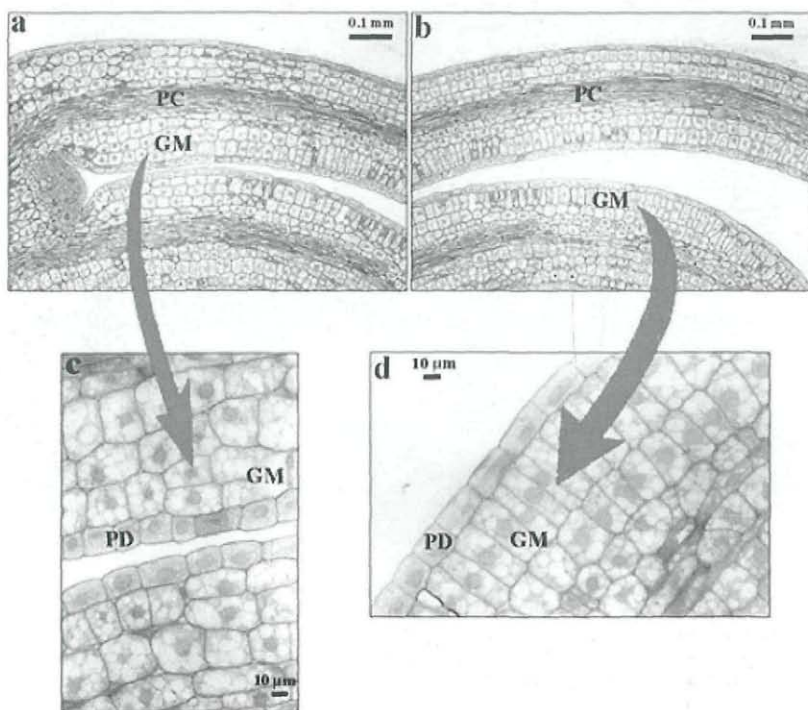


Fig. 2. Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 0. a. shoot apex region (GM = ground meristem; PC = procambium); b. cotyledonary region farther away from the shoot apex (GM = ground meristem; PC = procambium); c. cells in the ground meristem of cotyledon near the shoot apex (PD = protoderm; GM = ground meristem); d. cells in the ground meristem of cotyledon farther away from the shoot apex (PD = protoderm; GM = ground meristem).

The putative somatic embryo structures turned green 5-7 days after the explants were transferred to germination or conversion medium. After 7-10 days, they developed into plantlets. However, newly formed plantlets had never been found emerging from the hypocotyl of immature zygotic embryo explant.

Histology of somatic embryogenesis

At day 0, two main cell types were found underneath the protoderm of an immature zygotic embryo explant: cuboidal or rectangular cells which were near the shoot apex (Figs. 2 a and c) and columnar-shaped cells (Figs. 2 b and d) which were farther away from the shoot apex. Overall, the cotyledons of immature zygotic embryo explant exhibited typical anatomy of those of dicotyledoneous plants.

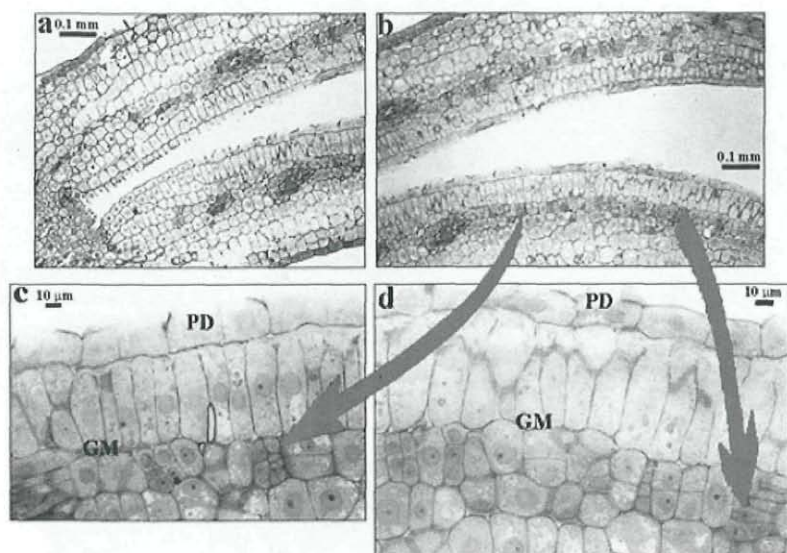


Fig. 3. Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 3. a. shoot apex region; b. cotyledonary region farther away from the shoot apex; c. and d. dividing cells in the different part of ground meristem of the cotyledon (PD = protoderm; GM = ground meristem).

At 3-5 days on induction medium, the cells underneath the protoderm in some parts of cotyledon were deviding periclinally (Figs. 3 and 4). At day 7, cell division in the same region occurred in both periclinal and anticlinal directions resulting in more cells (Figs. 5 a and b). This pattern of cell division associated with pepper somatic embryogenesis is different from that in other plant species.

For example, in the epidermal cells of *Trifolium repens* hypocotyl (MAHESWARAN & WILLIAMS 1985) and in the epidermis and subepidermis of the cotyledon explant of *Camellia japonica* L. (BARCIELA & VIEITEZ 1993) irregular, periclinal and oblique quantal divisions occurred during somatic embryogenesis. It has also been reported that only anticlinal cell divisions occurred in the epidermal cells of geranium hypocotyl that formed somatic embryos (HUTCHINSON & al. 1996).

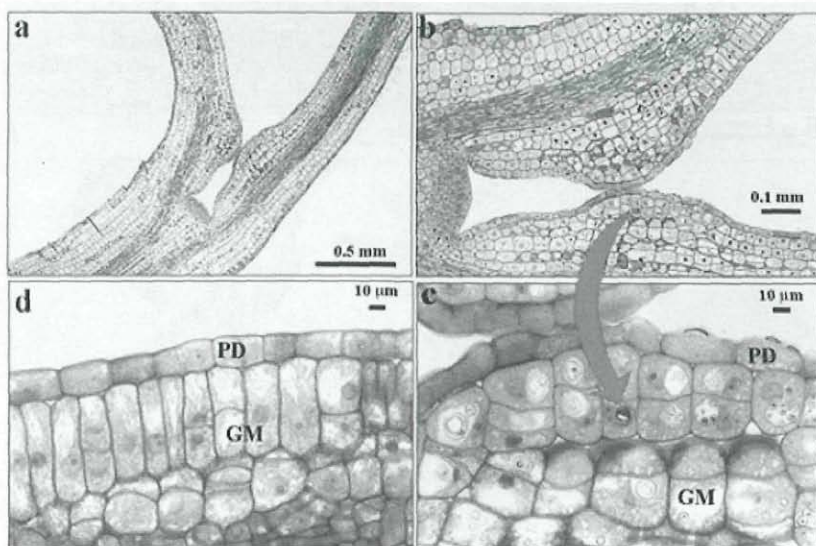


Fig. 4. Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 5. a. shoot apex region; b. somatic embryo structures developing near shoot apex; c., d. and e. dividing cells in ground meristem (GM) of the cotyledon (PD = protoderm).

The protoderm of the cotyledon was seen protruding due to increased cell division therein. Somatic embryo structures which were globular and lying on the inner surface of cotyledons could be found at around 10-16 days on induction medium (Figs. 5 c-e). These structures were similar to the globular somatic embryos of *Medicago sativa* L. and geranium described previously by DOS SANTOS & al. 1983 and HUTCHINSON & al. 1996, respectively.

Various sizes of them were seen developing at multiple locations on the cotyledons of the same immature zygotic embryo explant. On germination or conversion medium, the globular somatic embryo structures developed more into what appeared to be heart, torpedo and cotyledonary stages (Fig. 6) as previously described by DOS SANTOS & al. 1983 and HUTCHINSON & al. 1996.

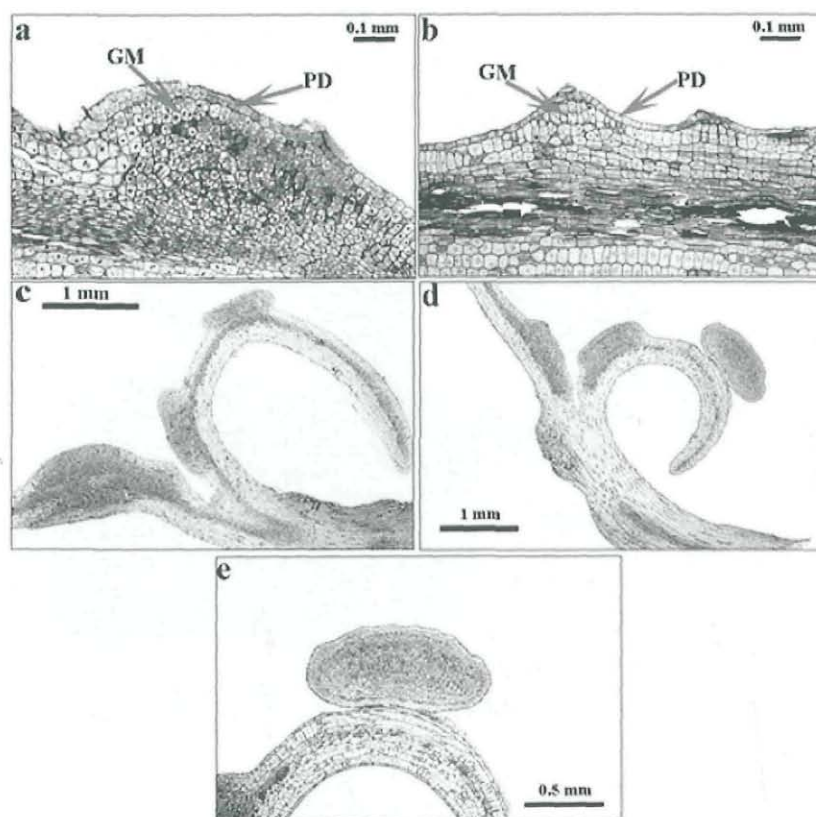


Fig. 5. Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium. a. and b. cells dividing in both periclinal and anticlinal directions in ground meristem (GM) of the cotyledon at day 7 (PD = protoderm); c. globular structures developed on the cotyledons at day 10; d. and e. globular structures developed on the cotyledons at day 16.

In summary, it has become clear from the present histological study that the primary event of interests occurring in the cotyledons of immature zygotic embryo explants on the somatic embryo induction medium was the formation of globular somatic embryos. After transfer to germination or conversion medium, not only somatic embryo germination or plantlet conversion was realized, but further development and maturation of the globular somatic embryos also took place. The use of the name for this medium following widespread practice in the literature is clearly unsatisfactory and might have contributed to some unsubstantiated claims (without accompanying histological evidence) that somatic embryo initiation and

maturation took place during culture on the somatic embryo induction medium (HARINI & LAKSHMI SITA 1993, BINZEL & al. 1996).

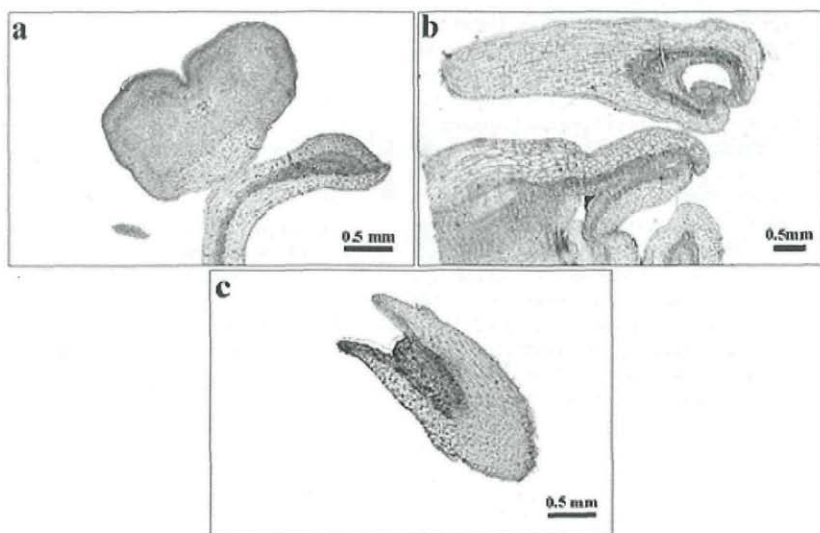


Fig. 6. Histology of immature somatic embryo of *Capsicum annuum* L. cv. Sweet Banana on conversion medium. a. a heart-shaped somatic embryo developed on the cotyledon at day 5; b. a torpedo-shaped somatic embryo developed on cotyledons at day 7; c. a cotyledonary-stage somatic embryo developed at day 10.

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