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THE EFFECT OF EXPLANT TYPE ON SOMATIC EMBRYOGENESIS INDUCTION IN *PISUM SATIVUM* L.

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K e y w o r d s: pea (*Pisum sativum* L.), somatic embryogenesis, somatic embryos.

Abbreviation: DAF – Days after flowering.

A b s t r a c t

The effect of explant type on somatic embryogenesis induction in *Pisum sativum* (cv. Oskar and an unregistered line HM-6) was studied. Shoot apices, leaf primordia, and epicotyl fragments of axenically grown, etiolated seedlings, as well as embryonic axes and cotyledon fragments isolated from zygotic embryos at different stages of development, were used as explants. Somatic embryogenesis was induced essentially as described by Griga in 1998 – MS salts and sucrose, B5 Gamborg vitamins, picloram (2.5 μM). After induction period (14 days) all cultures were transferred to the differentiation medium (basal medium as above, auxin omitted). Both in Oskar and HM-6, only shoot apices developed somatic embryos and (with significantly lower frequency) adventitious shoots.

WPŁYW RODZAJU EKSPLANTATU NA INDUKCJĘ SOMATYCZNEJ EMBRIOGENEZY U *PISUM SATIVUM* L.

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S ł o w a k l u c z o w e: groch, somatyczna embriogeneza, somatyczne zarodki.

Skróty: DPK – dzień po kwitnieniu.

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Abstrakt

W pracy zbadano zdolność wybranych rodzajów eksplantatów grochu odmiany Oskar oraz linii HM-6 do indukowania somatycznej embriogenezy. Jako eksplantaty zastosowano wierzchołki pędu, zawiązki liści i fragmenty epikotyli izolowane z aksenicznie hodowanych, etiolowanych siewek, jak również osie zarodkowe i fragmenty liścieni pobierane z zarodków zygotycznych na różnych etapach ich rozwoju. Somatyczną embriogenezę pobudzano w zasadzie wg procedury opisanej w 1998 r. przez Grigę, tj. na pożywce zawierającej sole i sacharozę jak w zestawie MS, witaminy zestawu B5 Gamborga oraz pikloram (2.5 μM). Po okresie indukcji (14 dni) wszystkie hodowle przenoszono na pożywkę różnicującą (podłoże podstawowe jak wyżej, ale bez auksyny). Zarówno u odmiany Oskar, jak i linii HM-6 zarodki somatyczne oraz (z istotnie niższą wydajnością) pędy przybyszowe uzyskiwano jedynie z wierzchołków pędów młodych siewek.

Introduction

Somatic embryos have been obtained from *in vitro* cultures of virtually all economically important plant species. However, various species, as well as cultivars (PODWYSZYŃSKA et al. 1997) and lines (GAIN et al. 1998) differ widely in embryogenic potential and requirements. The choice of explant (and nutrient medium, as well) is a critical factor determining the success of SE induction. Fragments of young seedlings and immature zygotic embryos are generally considered the most suitable explant sources (LAKSHMANAN and TAJI 2000, HITA et al. 2003, BELMONTE et al. 2007). Fragments of flowers can also be very embryogenic (PINTO-SINTRA 2007, SINGH et al. 2007, STEINMACHERA et al. 2007) and in bulbiferous plants somatic embryogenesis can be induced on fragments of bulb scales (eg. DUQUENNE et al. 2006).

Somatic embryogenesis in *Pisum sativum* L., unlike other legumes, is difficult to induce (LAKSHMANAN and TAJI 2000). With this in mind, we tested the effect of explant type on somatic embryogenesis induction in pea. Explants successfully applied for SE induction in other legumes were considered.

Materials And Methods

Plant Material

To obtain axenically growing seedlings, seeds were surface sterilized in 5% water solution of Chloramine B for 15 minutes followed by three washes with sterile distilled water. Disinfected seeds were placed in sterile tubes (25 dm^{-3} capacity) containing moist cotton wool. After germination (in darkness at 25–26°C for 4 days): shoot apices, leaf primordia and epicotyl fragments (0.1 cm long) were excised from seedlings, using a dissecting microscope, and placed on induction medium.

To obtain zygotic embryos, seeds were placed in pots filled with compost soil and sand (4:1, v/v). Seeds were regularly watered with tap water. The humidity of substrate was kept at 60–70%. Florovit fertilizer was applied three times: at the stage of five leaves, at the beginning of flowering and fruiting. Pods were collected 14, 18 or 22 days after flowering. Unopened pods were surface sterilized in 5% water solution of Chloramine B for 10–12 minutes followed by several washes with sterile distilled water. Embryonic axes and fragments of cotyledons (0.1 cm long) were isolated from zygotic embryos and placed on induction medium.

Seeds of *P. sativum* cv. Oskar and line HM-6 were kindly provided by AGRITECH Ltd. Czech Republic.

In vitro cultures

The explants were subjected to 14-days induction on basal medium described by GRIGA (1998), which contained MS salts (MURASHIGE and SKOOG 1962), Gamborg B5 vitamins (GAMBORG et al. 1968), 3% sucrose and 2.5 μM picloram. During the induction period cultures were kept in growth room under 16/8 hours (light/darkness) photoperiod at temperatures of 23–24°C at day and 19–20°C at night.

After induction all cultures were transferred to the differentiation medium (basal medium without phytohormones) and incubated in growth room at light/temperature conditions as given above. Three weeks later the physiological state of explants, morphogenetic responses of cultures and the efficiency of somatic embryogenesis were evaluated. Observations were repeated every 7–10 days. The efficiency of somatic embryogenesis was defined as number of somatic embryos per explant (expressed as per cent).

Statistical Analysis

One-way analysis of variance (ANOVA) was applied. The values analyzed were means of five series with four replicates within each series. Standard errors (± SE) were also determined. The obtained average values were separated using Duncan's multiple range test $P \leq 0.05$. The Microsoft Excel 2007 and STATISTICA 8.0 computer programs were used.

Results And Discussion

Among nine types/developmental stages of explants only one kind of explant, that is shoot apices, formed somatic embryos during differentiation culture. This is valid for both Oskar and HM-6, although these genotypes differed quite distinctly in the efficiency of somatic embryogenesis induction (Table 1) and the rate of embryo formation. In Oskar cultivar most embryoids appeared on explants after 25 days of culture, whereas in cultures of HM-6 line, somatic embryos appeared later, 35–45 days after explant isolation. Rather unexpectedly, no structures resembling somatic embryos developed on embryonic axes, although elongation and rooting of these explants could easily be observed (Table 2).

Table 1
The frequency of somatic embryogenesis and organogenesis on explants isolated from pea zygotic embryos or seedlings (mean ± S.E)

Cultivar	Explant	Somatic embryogenesis and regeneration efficiency [%]		
		somatic embryos	shoots	roots
Oskar	shoot apex	53.3 ±12.0^{a}	6.7 ± 3.3^{e}	20.0 ± 5.8^{d}
	leaf	0.0	0.0	36.7 ± 18.6^{e}
	epicotyle	0.0	0.0	16.7 ± 3.3^{df}
	embryonic axis 14 DAF	0.0	0.0	10.0 ± 5.8^{f}
	embryonic axis 18 DAF	0.0	0.0	0.0
	embryonic axis 22 DAF	0.0	0.0	26.7 ± 3.3^{d}
	cotyledon 14 DAF	0.0	0.0	0.0
	cotyledon 18 DAF	0.0	0.0	0.0
	cotyledon 22 DAF	0.0	0.0	0.0
HM-6	shoot apex	36.7 ± 6.7^{b}	6.7 ± 3.3^{e}	16.7 ± 6.7^{df}
	leaf	0.0	0.0	6.7 ± 3.3
	epicotyle	0.0	0.0	20.0 ± 5.8^{d}
	embryonic axis 14 DAF	0.0	0.0	20.0 ± 15.3^{d}
	embryonic axis 18 DAF	0.0	0.0	43.3± 12.0^{e}
	embryonic axis 22 DAF	0.0	0.0	36.7 ± 6.7^{e}
	cotyledon 14 DAF	0.0	0.0	23.3 ± 3.3^{d}
	cotyledon 18 DAF	0.0	0.0	0.0
	cotyledon 22 DAF	0.0	0.0	0.0

Values followed by the same superscript are not significantly different at 5% level (data in columns were compared).

Callus tissue developed on all explants, most rapidly during the first 30 days of culture, and covered about 10 to 100% of explants surface. The size and compactness of callus depended on explant type (Table 2). Embryonic axes formed soft callus, while on cotyledons it was compact with ragged surface and it was rigid and nodular on epicotyls. When embryonic axes of 18 or 22-days-

Table 2

Morphogenetic responses of explants after 60 days of *in vitro* culture

Explant		Responses	
		OSKAR	HM-6
Seedling	shoot apical	somatic embryogenesis, shoots, roots	somatic embryogenesis, shoots, roots
	leaf	roots	roots
	epicotyle	explant elongation, roots, green-white callus	explant elongation, roots, green callus
Zygotic embryo	embryo axis 14 DAF	bright-green callus, roots	bright-green or cream--yellow callus on hypocotyl and root, roots
	embryo axis 18 DAF	green callus	axis germination, root covered by callus
	embryo axis 22 DAF	roots	roots, necrosis
	cotyledon 14 DAF	cream-green callus on whole explant	roots
	cotyledon 18 DAF	cream-green callus covering whole explant	explants decay
	cotyledon 22 DAF	green callus	cream-green callus

-old seedling were used as explants, callus growth was very intense and it resulted in a four-fold increase of explant size. In case of embryonic axes isolated from 14-days-old seedlings callus proliferation increased approximately twice the explant size. The weakest morphogenetic reactions were observed on cotyledons isolated from 18-days-old zygotic embryos. Most of these explants formed only minute callus on the edges, and at 35 day of culture callus tissue started decaying. Necrosis was observed also on other explants, but less it covered less than 15% of their surface.

Rhizogenesis was observed on all types of explants, except cotyledons of immature zygotic embryos (18 and 22 DAF) – Table 2. In cultures of embryonic axes, roots developed from preexisting shoot apices, from the cells surrounding vascular bundles. Some adventitious roots were also formed on apical parts of explants. They reached 30–35 mm length and were covered by soft cream-bronze callus. In cultures of shoot apices roots developed in basal part of explants or in apical part of explant closely to developing shoot primordium. Adventitious roots were also formed by epidermal cells of epicotyles and cotyledons (14 DAF), as well as cells of leaf vascular bundles.

Various species, cultivars and genetic lines of legumes may differ widely in efficiency of somatic embryogenesis induction. Cultivar and genotype of explants cells affect both embryo formation but also its potential for vigorous growth (NADOLSKA-ORCZYK 1992, BRODA and TORZ 1997, GRIGA 1998, WALTER

and PARROT 2001, TOMLIN et al. 2002). The choice of explant, type of tissue and age of seedling used as explant source are considered critical factors that determine the success of somatic embryogenesis in legumes (GRIGA 1998, NADOLSKA-ORCZYK et al. 1994).

In our experiments with pea only shoot apices proved embryogenic. The suitability of this type of explant for somatic embryogenesis induction in pea was previously reported by LOISEAU et al. (1995) and GRIGA (1998). Leaves have not been used very often as explants in legumes, however they were suitable for *Cicer arietinum* (DINESHKUMAR et al. 1995), *Medicago arborea* (HITA et al. 2003) and *Medicago sativa* (MCKERSIE and BROWN 1996, RUDUŚ et al. 2000). Zygotic embryos or their fragments (especially cotyledons) have been successfully used as explant source by several authors studying legumes (HITA et al. 2003, BRODA and TORZ 1997, NADOLSKA-ORCZYK 2000, TOMLIN et al. 2002, GOGATE and NADGAUDA 2003). The stage of embryo development is considered an important factor for efficient SE induction (LOISEAU et al. 1996, RASHID 2001).

Conclusions

In our experiments, somatic embryogenesis could not be obtained either from axes or cotyledons of zygotic embryos at three stages of development/maturity (14, 18 and 22 DAF – Table 1). However, this does not seem to be a limitation typical for all *Pisum sativum* cultivars. Somatic embryogenesis induction on embryonic axes and cotyledons of *Pisum sativum* was achieved by NADOLSKA-ORCZYK et al. (1994) and LOISEAU et al. (1996).

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