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Propagation of *Chelidonium majus* L. by somatic embryogenesis

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Abstract

Direct somatic embryogenesis in celandine (*Chelidonium majus* L.) was achieved in epicotyl explants of seedlings after prolonged cultivation on Murashige and Skoog medium with or without plant growth regulators. Somatic embryos developed into plantlets which entered additional cycles of somatic embryogenesis. Cultures consisting of plantlets with prolonged embryogenic potential were maintained for five years on plant growth regulator free medium. Embryos which developed into rooted plantlets could be acclimated in a glasshouse enabling thus a continuous propagation scheme to be established.

Additional key words: celandine, growth regulator-free medium, medicinal plant.

Introduction

Celandine is a biennial or perennial herb growing as a weed in areas of human habitation. All parts of the plant produce poisonous, orange colored latex rich in alkaloids including: chelidonine, sanguinarine, chelerythrine, berberine, coptisine and others (Colombo and Tome 1995). In pharmacology, *C. majus* is known for its anti-microbial, antiinflammatory, spasmolytic and sedative effects. Celandine callus and cell cultures established by Colombo and Tome (1991, 1995) were used for study of alkaloid production and organogenesis. Indirect somatic embryogenesis of *C. majus* var. *asiaticum* was obtained from callus tissue of pedicels (Woo *et al.* 1996) and suspension cultures derived from immature ovules (Kim *et al.* 1999). There are no data for successful *in vitro* propagation of celandine based on axillary shoot proliferation.

Our investigation was performed with the aim to establish celandine shoot cultures which apart from propagation could also be used for studies of latex production. Epicotyl explants excised from seedlings failed to undergo proliferation of axillary shoots. Instead, some of them after prolonged subculturing entered direct somatic embryogenesis pathway. We here report on the embryogenic capacity of celandine epicotyl explants and a continuous propagation scheme based on secondary somatic embryogenesis.

Materials and methods

Seeds of celandine (*Chelidonium majus* L., *Papaveraceae*) collected at Kalemegdan location in Belgrade were surface sterilized for 20 min in commercial bleach containing 0.5 % NaOCl. Seeds were thoroughly rinsed in autoclaved water and germinated in Petri plates containing Murashige and Skoog's (1962) medium supplemented with 2 % (m/v) sucrose and 0.64 % (m/v) agar (basal, growth regulator-free medium). Medium pH was adjusted to 5.8 prior to autoclaving performed for 20 min at 114 °C. Temperature in the growth room was 25 ± 2 °C and a 16-h photoperiod with irradiance of 33 - 45 μmol $m^{-2}s^{-1}$ produced by cool white fluorescent tubes.

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Abbreviations: BA - 6-benzyladenine; GRF - growth regulator free; IBA - indole-3-butyric acid; Kin - kinetin; SE - somatic embryogenesis.
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Seed germination required a short 4 - 5 day chilling treatment in the refrigerator at 5 - 8 °C. Epicotyl explants were excised from 21-d-old plantlets and placed in 100-cm^3 Erlenmeyer flasks with 40 cm^3 solid Murashige and Skoog's (1962) media supplemented with: *1*) 0.2 mg dm^{-3} 6-benzyladenine (BA) and 0.1 mg dm^{-3} indole-3-butyric acid (IBA), *2*) 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA, *3* and *4*) 0.1 or 0.5 mg dm^{-3} kinetin (Kin), and *5*, *6*, *7*, *8*) with growth regulator-free (GRF) medium with 2, 4, 6 and 8 % sucrose, respectively. Each of these treatments consisting of 50 - 60 epicotyl explants followed through 7 subsequent 35 d long subcultures was replicated at least three times. Embryogenic cultures were further maintained in Petri plates (100 mm in diameter) with 25 - 30 cm^3 of GRF medium (*5*). Effect of sucrose and BA on secondary somatic embryogenesis was investigated using single 1 - 2 mm long embryos as explants. Treatments consisting 60 - 80 embryos were replicated at least twice. Statistical analysis of data was performed using Duncan's multiple range test.

For histological studies explants were fixed in FAA [40 % formalin (5 cm^3) : acetic acid (5 cm^3) : 70 % ethanol (90 cm^3)] for 24 h, dehydrated in ethanol series and embedded in paraffin. Sections 10 - 15 μm thick were cut on a rotary microtome, stained with Delafields haematoxyline and mounted in Canada balsam (Johansen 1940).

Rooted plantlets 5 - 10 mm long were planted in trays with commercial peat based substrates and further grown in a glasshouse. Plants were initially (first two weeks) protected from desiccation using thin transparent plastic foil as a cover over trays.

Results

On all eight types of media epicotyl explants expanded forming a leafy rosette but none of them manifested axillary shoot activation or proliferation. Only explants on GRF medium formed adventitious roots. After 3 - 4 weeks there was no further growth of explants. Cessation of growth was roughly concomitant with increased yellow discoloration of the medium. The explants which appeared healthy were subcultured to fresh medium of the same composition. However, after recovering some of the tissue and leaves lost by trimming explants again stopped growing. By the end of the second subculture somatic embryogenesis was observed in several explants on GRF and kinetin supplemented media (Table 1).

Table 1. Somatic embryogenesis and morphogenesis of epicotyl explants, measured after 7 subsequent subcultures.

Growth regulators [mg dm^{-3}]	Number of explants	Somatic embryogenesis [%]	Differentiation
BA 0.2 + IBA 0.1	130	7.7	none
BA 0.5 + IBA 0.1	129	6.1	none
Kin 0.1	122	1.7	none
Kin 0.5	123	13.1	none
GRF + 2 % sucrose	125	9.6	roots
GRF + 4 % sucrose	129	3.1	roots
GRF + 6 % sucrose	111	3.6	roots
GRF + 8 % sucrose	101	0	none

Embryos appeared directly from the basal portions of explants which were in contact with medium but without formation of intervening callus tissues. The number of explants which entered embryogenesis increased in every new subcultures whilst the total number of initial epicotyl explants decreased due to necrosis in every new subculture. After eight subcultures all epicotyl explants in all treatments perished from necrosis apart from those which entered somatic embryogenesis. Primary somatic embryos were excised and subcultured to fresh GRF medium. Here embryos either rapidly developed into mature, rooted plantlets or produced new somatic embryos entering secondary somatic embryogenesis pathway.

Sometimes even large, rooted plantlets had secondary somatic embryos attached to their shoots, roots or leaves (Fig. 1*a*,*b*,*d*). Cultures comprising a mixture of somatic embryos at various stage of development (Fig. 1*e*) have been continuously maintained by subculturing on GRF medium for 5 years. Plantlets derived from these cultures produced orange latex. Development of plantlets was not arrested by media discoloration. Their roots had a characteristic dark brown, nearly black color. Rooted plantlets 10 mm or more in length (Fig. 1*c*) were easily acclimated in a glasshouse where they grew into normally appearing plants.

Histological examination showed embryo initiation to occur in surface layers of plantlets and embryos. In the earliest stage of embryo formation epidermal cells create a small surface protuberance (Fig. 2*a*) consisting of several meristematic cells with dense cytoplasm and prominent nucleus shifted towards the surface of the explant. At these locations embryos develop into individual structures well delimited from the neighboring tissue (Fig. 2*b*). All stages of embryo development including globular (Fig. 2*d*), heart (Fig. 2*c*), torpedo and cotyledonary (Fig. 2*e*) were observed. Many embryos were loosely connected or completely free from explants (Fig. 2*c*). New embryos frequently appeared as protuberances on the surface of other more developed embryos (Fig. 2*d*).

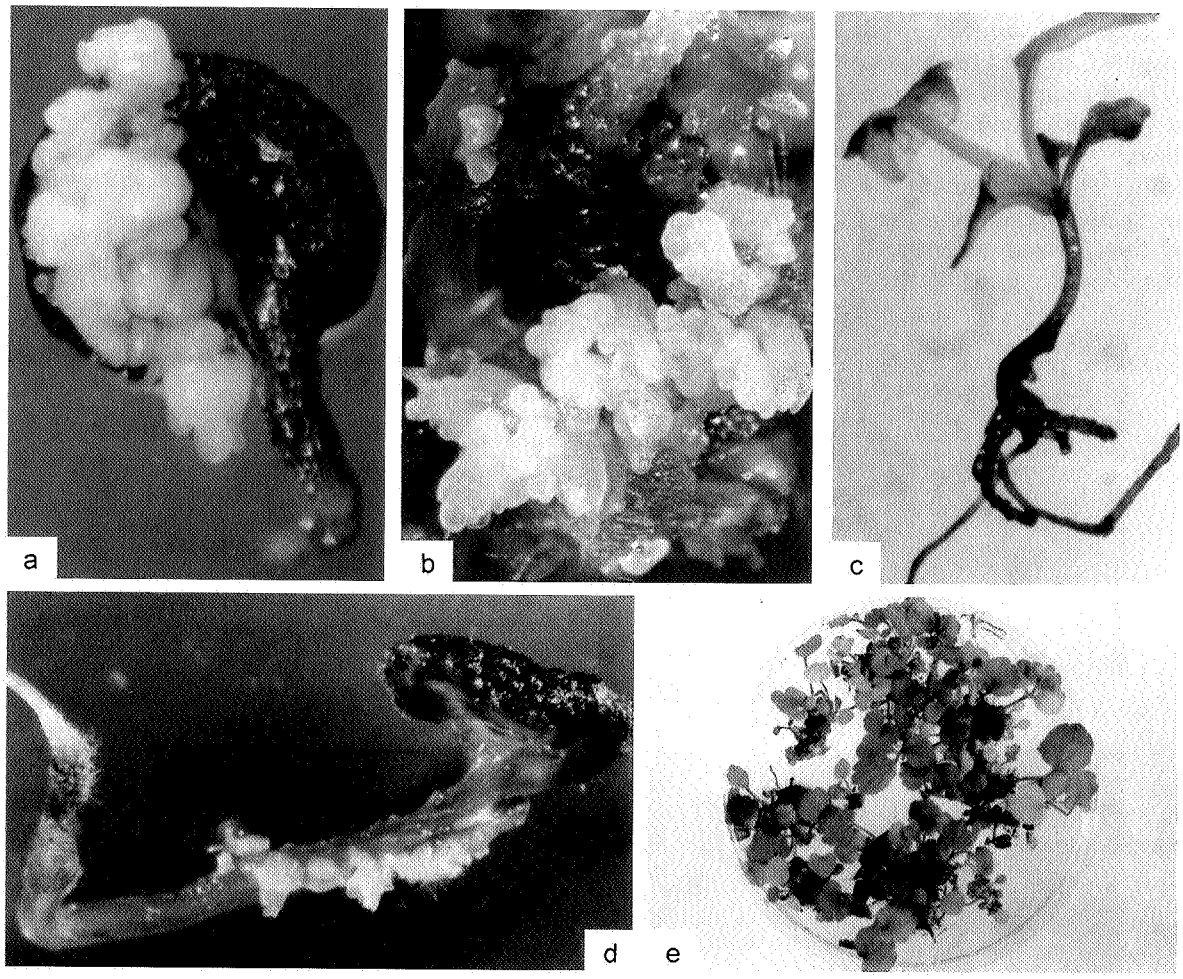

Fig. 1. *a* - somatic embryos developing directly on a cotyledon of a small, necrotic embryo, *b* - typical secondary somatic embryogenesis, *c* - large, rooted plantlet ready for transfer to soil, *d* - somatic embryos developing on a hypocotyl of a plantlet with bended root, *e* - Petri plate cultures comprising mixture of somatic embryo plantlets of various size.

Table 2. Effect of BA and sucrose on secondary somatic embryogenesis (SSE). Means ± SE, n = 120 - 180. Within each colum means followed by the same letter were not significantly different according to Duncan's multiple range test at $P \geq 0.05$.

BA [mg dm^{-3}]	Sucrose [%]	Cultures with SSE [%]	SSE per culture	Embryo length [mm]	Necrotic explants [%]	Plantlets more than 5 mm long [%]
0	2	37.4	2.8 ± 0.5 ^{a}	4.2 ± 0.1 ^{c}	48.8	8.5
0	4	38.3	3.1 ± 0.8 ^{ab}	3.9 ± 0.2 ^{bc}	44.2	6.6
0	6	41.9	6.0 ± 1.2 ^{b}	3.4 ± 0.2 ^{abc}	52.4	5.6
0.1	2	36.4	1.3 ± 0.3 ^{a}	3.6 ± 0.2 ^{ab}	22.7	18.4
0.5	2	43.6	0.8 ± 0.1 ^{a}	3.4 ± 0.2 ^{ab}	23.1	10.3
1.0	2	20.0	0.4 ± 0.1 ^{a}	3.2 ± 0.2 ^{a}	2.5	7.5

The effect of BA and sucrose on secondary somatic embryogenesis was investigated using small 1 - 2 mm long somatic embryos (Table 2). Production of new embryos was increased by sucrose but decreased by BA as compared to the basal GRF medium. Also, elongation of secondary embryos decreased upon addition of either BA or sucrose. However, addition of BA strongly suppressed necrosis of explants supporting at the same

time development of embryos into plantlets. Highest percentage of embryos developing into plantlets (18.4 %) was recorded on medium with 0.1 mg dm^{-3} BA. Percentage of cultures undergoing secondary embryogenesis in all treatments was around 40 % except for the highest BA concentration (1.0 mg dm^{-3}) where it was twice lower.

Finally, micropropagation was attempted again this time using apical explants from well developed plants derived from somatic embryos. In contrast to explants derived from zygotic embryos explants derived from somatic embryos grew well on medium supplemented with 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA manifesting reasonable shoot multiplication. Cultures appearing as short, rosette type clusters have been maintained for more than a year by regular subculturing at 4 - 5 week intervals.

Efficiency of acclimation for rooted plantlets 5 - 10 mm long was 84 %.

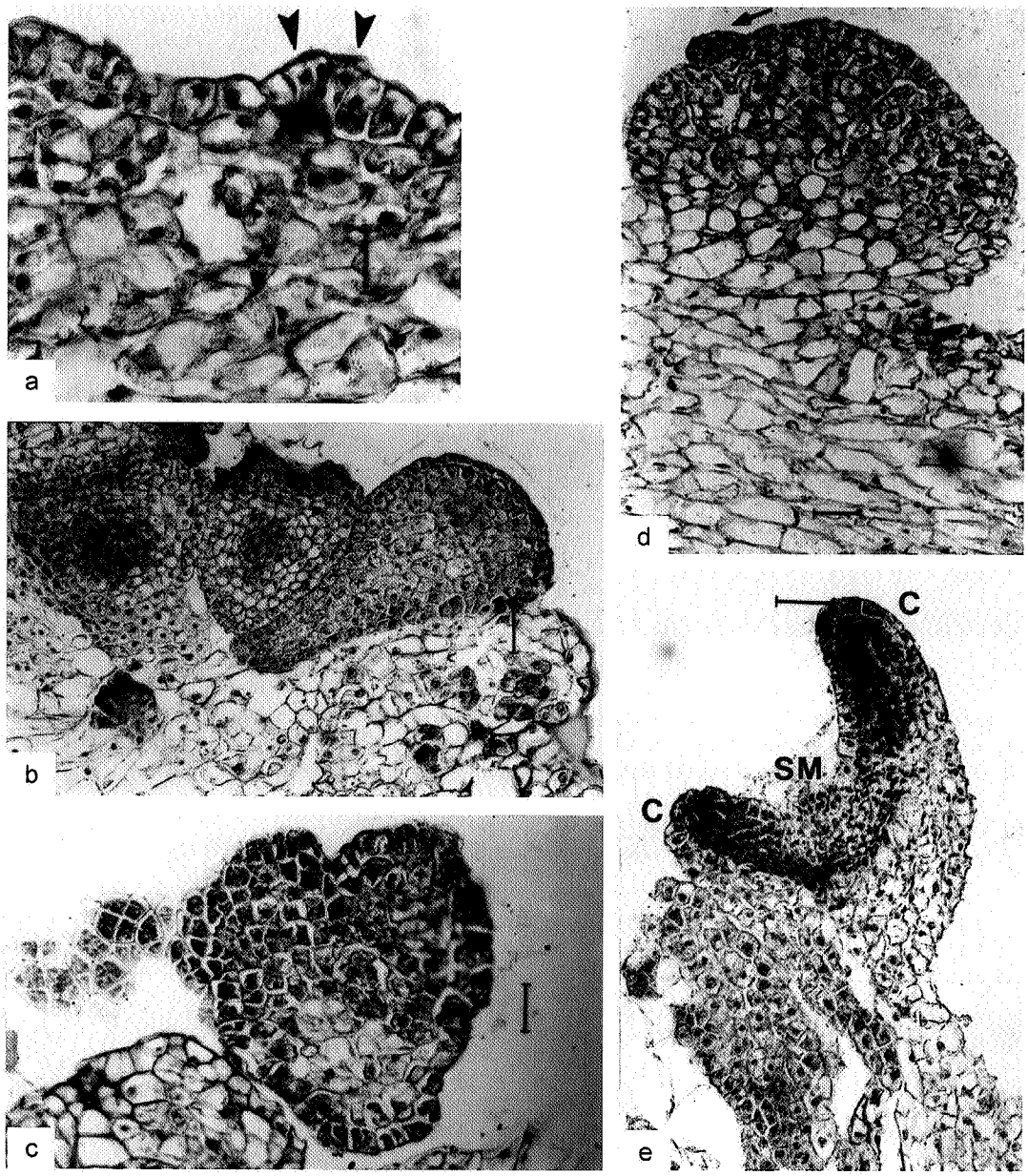

Fig. 2. *a* - initial stage of SE formation in epidermal tissue, *bar* = 45 μm, *b* - group of well delimited SE at various stages of development, *bar* = 60 μm, *c* - detached globular and heart shaped embryos, *bar* = 45 μm, *d* - globular SE, *arrow* indicates position of new (secondary) embryo formation, *bar* = 45 μm, *e* - cotyledonary stage embryo with well expanded cotyledons (C) and apical shoot meristem (SM), *bar* = 60 μm.

Discussion

According to Krikorian (1982), extended somatic embryogenesis on growth regulator-free medium in plants can be considered as a universal feature. Apart from carrot, secondary (recurrent, repetitive) somatic embryogenesis on growth regulator-free media has been observed in a number of species (Parrot and Bailey 1993). In these species, an inductive factor, usually a growth regulator or yeast extract is required to start embryogenesis which can then be repeated or continuously perpetuated on medium containing no growth regulators. In celandine we showed that somatic embryogenesis can be triggered on plant growth regulator-free medium, apparently in absence of a specific inducing factor. *Camellia japonica* is another species which can enter somatic embryogenesis on plant growth regulator-free medium (Vieitez *et al.* 1991).

There is a possibility that wounding *per se* could be the inductive stimulus for somatic embryogenesis in epicotyl explants of celandine and perhaps other species. Thus in carrot Smith and Krikorian (1990) showed that wounding of zygotic embryos serves as an inductive factor for somatic embryogenesis. In celandine, the percentage of explants entering somatic embryogenesis clearly increased with the number of subcultures performed.

Frequency of SE which we obtained using epicotyl explants on GRF medium (8.2 %) and 0.5 mg dm^{-3} K (13.1) was much lower than the frequency which (Kim *et al.* 1999) reported for ovule explants on 2,4-dichlorophenoxyacetic acid supplemented medium (40 %). Important difference between these two procedures is that in ovule explants somatic embryogenesis was indirect (from callus) whilst in epicotyl explants embryos formed directly from explants without production of callus. However, since in our scheme nearly 30 % of single isolated embryos developed into rooted plantlets and since the percentage of acclimated plantlets was higher than 84 %, we can recommend this procedure for propagation purposes.

The most intriguing finding in our report was the difference in the growth capacity of apical shoot explants excised from plants derived from zygotic and somatic embryos. Explants from zygotic embryos failed to manifest any growth responses on a variety of media, some of which are commonly used for micropropagation of many different plants species. Explants of the same type but excised from somatic embryos manifested good growth potential developing either in rooted plantlets (GRF medium) or shoot cultures with proliferating axillary buds (cytokinin supplemented medium).

Celandine micropropagation thus seems to be possible but only employing explants derived from somatic embryos. Obviously there is no need to insist on propagation via axillary shoot proliferation when somatic embryogenesis already offers a suitable propagation scheme.

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