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CELL BIOLOGY AND MORPHOGENESIS

Auxin pulse treatment holds the potential to enhance efficiency and practicability of somatic embryogenesis in potato

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Abstract The objective of the current study was to simplify existing somatic embryogenesis systems in potato (*Solanum tuberosum* L.) cv. Desiree. The project targeted the agar-based induction phase of the potato somatic embryogenesis process as the key area for improvement. Experiments were established to ascertain the effect of a 2,4-D (2,4 dichlorophenoxyacetic acid) pulse, applied to the primary internodal section explant source and its subsequent effect on embryo induction. Parameters tested were the duration of the auxin pulse in a range from 0 to 300 min, and the concentrations of 2,4-D applied, in a range from 0 to 5,120 μM. The mean number of somatic embryos formed per explant was recorded after 4 and 8 weeks culture. Our findings indicated that the somatic embryogenesis in potato internodal segments could be evoked by an auxin (2,4-D) pulse treatment over a wide concentration and duration range. The results further suggested that a simple 20 μM 2,4-D pulse treatment could replace a lengthy 2 week induction phase in potato somatic embryogenesis and thus improve the system's practicability for wider uptake.

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Abbreviations

2,4-D	2,4 Dichlorophenoxyacetic acid
cDNA	Complementary DNA
GA_3	Gibberellic acid
IAA	Indole acetic acid
MS	Murashige and Skoog
NAA	Naphthalene acetic acid

Introduction

Achievements in somatic embryogenesis, including its application to an increasingly wide range of species, have progressed with the continuous development of effective media formulations and methodology improvements. Somatic embryogenesis protocols generally employ the sequential use of two media compositions, an induction medium followed by a somatic embryo regeneration medium (Krikorian 2000). In many protocols, the induction medium contains auxin, usually 2,4-D, which has been widely accepted to be the principal controlling factor for embryogenesis. However, some workers have also reported the use of cytokinins in induction medium (Iantcheva et al. 1999). The regeneration medium usually lacks auxin, or contains very small concentrations, but is often supplemented with other additives such as gibberellins and abscisic acid, which are recognised for their beneficial effects on embryo development and maturation (Brown et al. 1995). At the time of culture initiation, the ability to synthesise auxin and cytokinin is lacking in cultured cells/tissues (Xing et al. 2000). Therefore, an empirical approach

is generally adopted for the optimisation of conditions for inducing somatic embryogenesis. This often involves analysing the effect of different media and culture conditions, coupled with an optimisation of the type and levels of plant growth regulators.

Despite the progress made in understanding somatic embryogenesis, since it was first reported in carrot (Steward et al. 1958), the changes that a somatic cell must undergo to become embryogenic are still not clear. Though it is apparent that there is no single and universally applicable signal that turns somatic cells embryogenic, it is generally accepted that plant growth regulators play the most significant role. In this context, auxin is regarded as the most important plant growth regulator in controlling somatic embryogenesis (Cooke et al. 1993). Both the endogenous auxin levels, and their exogenous application can be considered as critical factors during the induction and expression of somatic embryogenesis.

In the majority of plant species, the synthetic auxin 2,4-D is the most commonly used plant growth regulator for inducing somatic embryogenesis. Culturing explants under the influence of 2,4-D results in the increase in endogenous auxin levels in explants (Michalczuk et al. 1992) thus increasing cell division and developing the hormonal gradient necessary for gaining embryogenesis. Once the somatic cells are embryogenically induced, further expression of somatic embryogenesis may be initiated by a number of factors, depending on plant species, cultivar type and physiological condition of the donor plant. The most commonly observed of these is the elimination or reduction of the 2,4-D concentration applied.

Embryogenesis can be a complex multi-stage process, and in the important crop plant potato (*Solanum tuberosum* L.) the system involves two media changes and a lengthy time period (>9 weeks) for the full expression of somatic embryogenesis (Seabrook and Douglass 2001; Sharma and Millam 2004). In addition to reducing the length of time of the process in order to expedite embryo production for a wide range of fundamental or applied applications there is a requirement to avoid, or at least minimise the potential occurrence of somaclonal variation. To facilitate this, the use of media with the minimum addition of plant growth regulators would be preferred, or alternatively, a minimisation of the exposure period to plant growth regulators. A number of strategies for delivering a precise or temporal dosage of auxin at a key developmental stage could possibly be devised, but would only be applicable to a liquid-based system. For agar-based systems, possibly the most appropriate strategy would be the use of a Pulse-treatment, as previously reported, for example, by Dudits et al. (1991) using alfalfa and Kitamiya et al. (2000) using carrot. Solanaceous plants were considered to be generally recalcitrant for somatic embryogenesis potential (Litz and Gray 1995), with the exception of the non-tuber bearing species *Solanum melongena* where a high frequency of embryogenesis was first described by Gledddie et al. (1983) and the topic reviewed by Kantharajah and Golegaokar (2004). In potato, however, where the potential of an embryo-based regeneration system for high volume multiplication in potato seed technology and for use in transformation systems is high, there have been only limited reports on somatic embryogenesis. A system that took over 6 months to produce embryos in one cultivar was described by de Garcia and Martinez (1995) and this time period was reduced and expanded to a wider range of germplasm by Seabrook and Douglass (2001) and to a popular Indian cultivar by JayaSree et al. (2001). The use of a cell suspension system was reported by Vargas et al. (2005) and the first definitive histological evidence of the process was demonstrated by Sharma and Millam (2004). None of the workers have so far reported the use of a pulse treatment approach in potato and a series of experiments were devised in this current study to investigate the potential of this interventional approach.

Materials and methods

Plant material

Potato cv. Desiree (source material originally obtained from the Scottish Agricultural Science Agency, Edinburgh) in vitro cultures were maintained and multiplied on Murashige and Skoog (1962) medium, without plant growth regulators, using single node cutting explants in VitroVent containers (Duchefa). The cultures were incubated under controlled environmental conditions maintained at 19 ± 1°C, 16/8 h light/dark cycles and 90 μmol/m^2/s photon flux density (400–700 nm).

Determining the temporal requirement and optimum concentration of the 2,4-D pulse required for the induction of embryogenesis competence

Internodal sections from 4 to 6 weeks old cultures were excised and immersed in 0, 1.25, 5, 20, 80, 320, 1,280 and 5,120 μM 2,4-D solutions prepared in stage-1 liquid medium (Sharma and Millam 2004) which was filter-sterilised through a 0.22 μm filter. The explants were incubated under constant orbital shaking (~75 rpm) conditions for 1 h at 19 ± 1°C. Following this pulse treatment, the explants were given six sterile washings using sterile MS liquid medium and cultured on modified MS medium plates supplemented with 0.05 μM IAA, 12.0 μM Zeatin and 0.55 μM GA_3 (Sharma and Millam 2004).

From the results of the experiments described above, the optimised 2,4-D pulse concentrations were then tested over different pulse durations (0, 0.5, 30, 60, 120, 180 and 300 min) to further investigate the enhancement of somatic embryogenesis. Following the pulse treatment, all explants were then immediately cultured on stage-3 (Sharma and Millam 2004) medium and incubated as described. The mean number of somatic embryos formed per explant was recorded visually after 4 and 8 weeks in culture. Histological analysis of the explants was as described by Sharma and Millam (2004). The experiments were replicated three times and each replicate consisted of five internodal explants per treatment. Data storage and calculations were done using the Genstat 7 statistical package (Payne et al. 1993). The data were analysed by analysis of variance (ANOVA) and the individual group means were ranked by comparing their mean differences against LSD (least significant difference).

Results and discussion

Previously in crops other than potato, it has been demonstrated that 2,4-D indirectly inhibits the elongation of cells by promoting cell division (Lloyd et al. 1980), stimulating asymmetrical cell division (Pasternak et al. 2002; Bögre et al. 1990) and affecting the regulation and balance of endogenous hormones (Xing et al. 2000). The use of 2,4-D for inducing somatic embryogenesis in potato has been previously advocated (de Garcia and Martinez 1995; JayaSree et al. 2001; Vargas et al. 2005; Sharma and Millam 2004) but none of these reports involved the pulse treatment approach. In the current study, 2,4-D was found to be more effective than IAA and NAA (data not shown) and was employed to overcome the complexities reported in previous potato somatic embryogenesis procedures.

Preliminary experimentation was performed to ascertain the degree to which each individual somatic embryogenesis stage (Sharma and Millam 2004) was essential. The first and third stages were found to be indispensable, due to facilitating the availability of explants and harvesting of somatic embryos, respectively. The exposure of the explants to some form of induction treatment (stage-2) was also found to be essential, as the culturing of fresh explants directly on stage-3 medium was found to be ineffective for evoking somatic embryogenesis. Maintaining the explants on stage-2 was also not effective for the production of somatic embryos, unless the induced explants were transferred to stage-3 medium. Thus, it was inferred that a three-stage (shoot multiplication, somatic embryogenesis induction and expression) regime was essential and efforts were made to further refine and decrease (or compress) the time scale involved in the somatic embryogenesis protocol by minimising the induction phase to the shortest possible in order to minimise exposure to 2,4-D while still stimulating somatic embryogenesis.

To achieve this, internodal segment explants of potato cv. Desiree were pulse-treated at different 2,4-D concentrations and time durations, and then immediately cultured on stage-3 medium. The results indicated that 2,4-D in the range from 1 to 320 μM was effective for inducing somatic embryogenesis in potato, beyond which it was found to be detrimental to the induction of embryogenesis (Fig. 1a). A 20 μM pulse was found to be significantly effective for embryo induction, followed by 80, 320 and 5 μM treatments. For optimising the pulse-exposure period, a 20 μM 2,4-D pulse was used for 0, 0.5, 30, 60, 120, 180 and 300 min durations. All durations, except the control (no treatment) were effective for evoking somatic embryogenesis, but a 20 μM pulse treatment for 1 h yielded a significantly higher number of somatic embryos (Figs. 1b, 2) as compared to all other durations attempted. These results are in agreement to Dudits et al. (1991), who found a 2,4-D pulse of 100 μM for 1 h sufficient to enable alfalfa microcallus cells competent for embryogenesis. Kitamiya et al. (2000) induced carrot somatic embryogenesis by pulse-treating hypocotyl segments with a high 2,4-D concentration (450 μM) for 2 h, and also identified a differentially expressed carrot cDNA (Dcarg 1) as a function of the embryogenic response.

The pulse data further reveals that the time duration required for achieving somatic embryogenesis in pulse-treated sets was approximately equal to the cumulative time period required for the separate induction (containing 2,4-D) and expression phases (without 2,4-D, embryo forming phase), described previously for a 3-stage somatic embryogenesis process. This further implies that, though a short 2,4-D pulse was able to provide the necessary stimuli for inducing somatic embryogenesis, a 2 week preparatory period was still required by the competent cells before entering into the embryo forming and transition stages. A similar finding was reported by Lad et al. (1997) in mango nucellar cultures. While they identified a 28 day exposure to 4.5 μM 2,4-D as optimum for gaining embryogenic competence in mango using a test pulse-treatment ranging from 0 to 63 days, the minimum time required for the differentiation of heart stage somatic embryos upon transfer of embryogenic cultures to semisolid medium was found to be ~72 days, irrespective of the length of pulse-treatment.

The combined inferences from both modified somatic embryogenesis procedure using pulse treatment as well as a standard 3-stage regime reveals that the initial embryo-

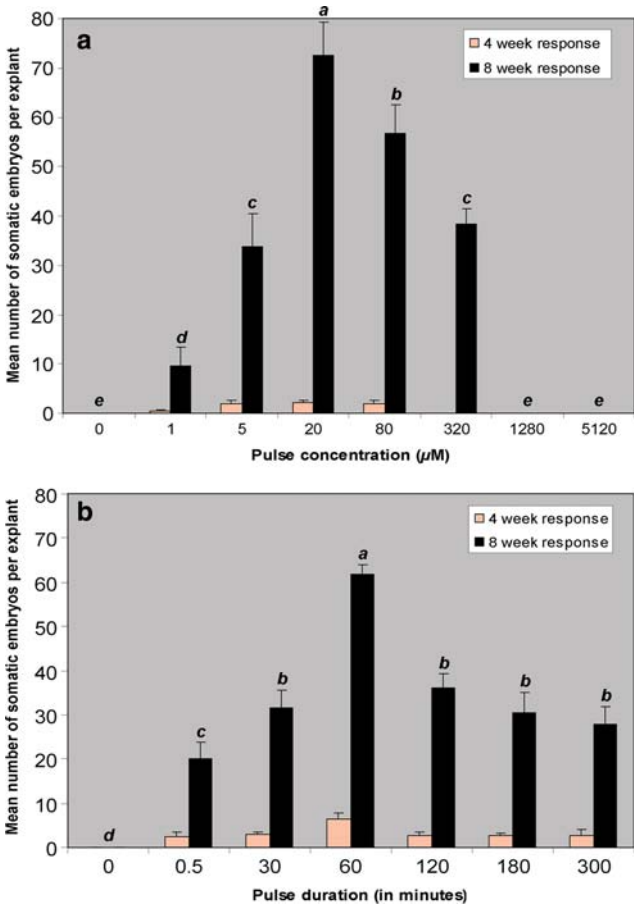

Fig. 1 Pulse experiment data. **a** Optimisation of pulse concentration using 1 h 2,4-D treatment; **b** optimisation of 2,4-D treatment time using a 20 μM pulse. *Error bars* show the standard error of the mean for three replicate measurements, each comprising five internodal segment explants. Treatment means corresponding to the histobars sharing the same letters are not significantly different at $P < 0.001$. The LSDs for pulse concentration and duration data were 12.06 and 9.752, respectively

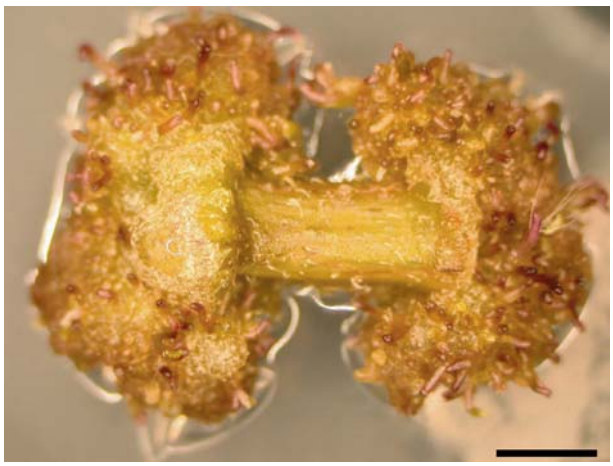

Fig. 2 Potato somatic embryos developing from a cv. Desiree internodal segment explant after 8 weeks of incubation on embryo regeneration medium following a 20 μM 2,4-D pulse treatment. Bar: 10 mm

genesis process after induction can progress in the absence as well as presence of 2,4-D beyond which the withdrawal of 2,4-D from the media becomes necessary. Thus, the success of an auxin pulse in evoking a somatic embryogenesis response indicates that exogenous auxin might only be needed for initiating the first step in the somatic embryogenesis cascade and further differentiation of induced cells to somatic embryos may proceed in the absence of exogenous auxin. The absence or presence of auxin in reduced amounts has been reported to be contributory to the activation of genes essential for the transition to the heart stage (Zimmerman 1993). This observation was further supported in the histological investigation carried out during the course of the current study. The histological analysis showed the presence/development of only pro-embryogenic-mass (Fig. 3a) in the 2,4-D exposed cultures without any organised structures, which were only apparent (Fig. 3b) after the removal of 2,4-D from the media. This led us to hypothesise that exposure of cells to an auxin-shock for a limited period could be sufficient to evoke somatic embryogenesis in potato cultures.

The auxin 2,4-D has been considered to regulate the endogenous IAA metabolism more appropriately as compared to other synthetic plant growth regulators for the induction of somatic embryogenesis. In carrot the gain of embryogenic response as a result of exposure to exogenous 2,4-D was also associated with a multi-fold increase in endogenous IAA levels, which further suggested that the synthetic auxin 2,4-D rather than having significant direct effects on somatic embryogenesis might be acting indirectly by disturbing the endogenous auxin metabolism (Michalczuk et al. 1992). A similar association of high endogenous IAA levels and achieving embryogenic competence has been reported in other plants such as alfalfa (Pasternak et al. 2002). It has been postulated that 2,4-D promotes the production of IAA binding proteins and increases the sensitivity of the cells to IAA (Lo Schiavo et al. 1991) rendering them embryogenically competent. Also, in carrot zygotic embryogenesis, an auxin surge has been reported to occur just after fertilisation (Ribnicky et al. 2002), which further emphasises the importance of temporal changes in endogenous auxin levels in the expression of cellular totipotency. These observations suggest that an endogenous auxin pulse may be one of the primary signals leading to somatic embryogenesis (Thomas et al. 2002), which under in vitro conditions could also be achieved by simple treatments such as an exogenous auxin pulse treatment.

Though the pulse treatment approach did not reduce the time period required for the whole process of somatic

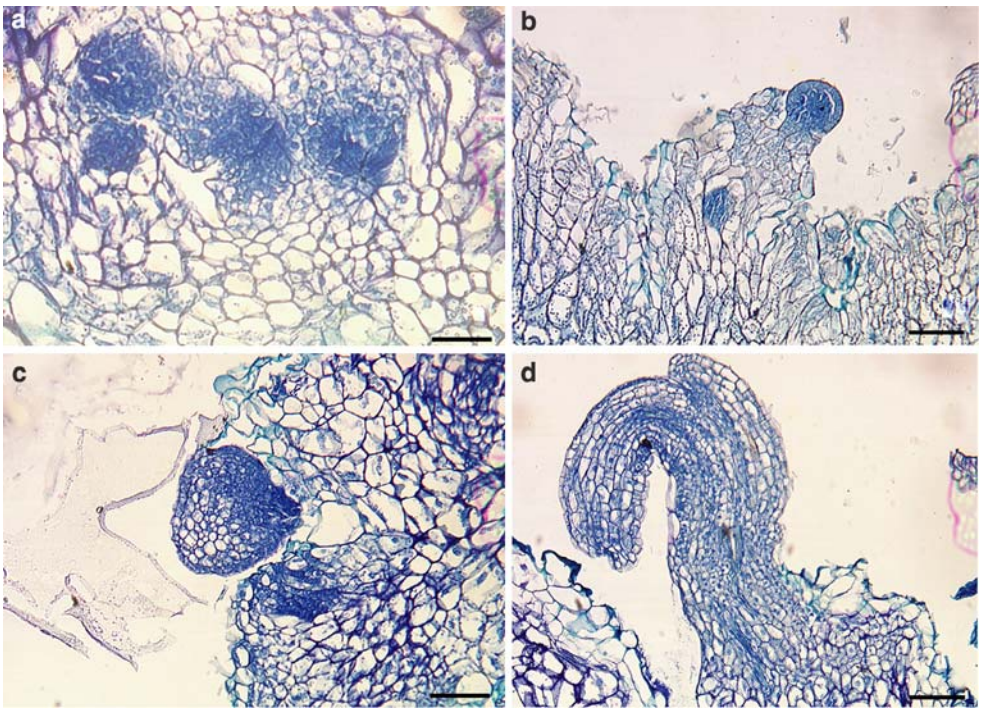

Fig. 3 Effects of 2,4-D treatment upon progression of somatic embryogenesis in potato. **a** Development of proembryogenic masses in the presence of 2,4-D; advancement of somatic embryogenesis through characteristic globular (**b**), heart (**c**) and torpedo (**d**) stages of embryogeny after removal of 2,4-D. Bars: a = 60 μm; b, c and d = 120 μm

embryogenesis in potato, in terms of the practical applicability of somatic embryogenesis in commercial operations the approach presented offers some potential. Somatic embryogenesis has been projected as an effective tool for mass multiplication of elite germplasm. Though liquid somatic embryogenesis systems are preferred over semi-solid methodologies for practical reasons, as agar based systems are labour intensive and resource inefficient, embling (a synonym to ''seedling'' for a plant obtained from a somatic embryo) quality tends to be better when derived using the latter approach. Notwithstanding, this can be improved and made more cost effective by omitting unnecessary steps and simplifying the process by employing novel approaches such as pulse treatment, which has not been applied for potato to date. The establishment of a reproducible and modified auxin pulse based somatic embryogenesis system provides a unique potato propagation system for the adoption of this technology into commercial practice with the added advantages that it would reduce the complexities, consumable requirements and number of subcultures which are significant aspects for commercial applications of this potentially useful technology.

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