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Significance of the zygotic seed coat on quiescence and desiccation tolerance of *Medicago sativa* L. somatic embryos

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Summary. The influence of the zygotic seed coat on precocious germination and desiccation tolerance of somatic embryos has been studied using alfalfa (*Medicago sativa* L.). When cultured in contact with somatic embryos, seed coats at certain developmental stages inhibited precocious germination and induced desiccation tolerance in the somatic embryos. Germination of somatic embryos was inhibited by seed coats at the age of 16-26 days after pollination (DAP) and desiccation tolerance was induced after 20-26 DAP. Both phenomena were related to the synthesis of abscisic acid in the seed coat. The absence of a quiescent phase and desiccation tolerance in alfalfa somatic embryos may be related to the lack of developmental control by the seed coat.

Keywords: somatic embryogenesis, artificial seeds, abscisic acid, stress tolerance

Abbreviations: ABA, Abscisic acid; DAP, Days after pollination.

Introduction

Somatic embryogenesis is considered to be a potential clonal propagation system of plants and the optimism that somatic embryos can be used as artificial seeds has increased in recent years (Attree and Fowke 1991; Attree et al. 1991; Gray and Purohit 1991; Redenbaugh 1990; Redenbaugh and Ruzin, 1989; Senaratna 1992). The physiology and development of seeds are being studied with the objective to apply such information to improve the quality of somatic embryos (Buchheim et al. 1989; Carman 1988; Coulter and Bewley 1990; Gray et al. 1987). A number of physiological and developmental similarities and contrasts exist between somatic and zygotic embryos (Gray and Purohit 1991; Senaratna 1992). Somatic embryos arise from somatic cells in response to a chemical signal (e.g. auxin), and essentially represent a clone of the parent plant. The zygotic embryo is a result of the sexual combination of male and female gametes. The developmental ontogeny for both types of embryos proceeds through globular, torpedo, and cotyledonary stages for dicots and conifers (Becwar et al. 1989), or globular, scutellar and coleoptillar stages for monocots (Gray and Purohit 1991).

Natural desiccation induces a quiescent phase with zygotic embryos. Alfalfa somatic embryos are not normally desiccation tolerant and, thus do not have a quiescent phase (Senaratna et al. 1989). However, desiccation tolerance can be induced in somatic embryos by external stimuli (Senaratna et al. 1989, 1990). For example, treatment of alfalfa somatic embryos with abscisic acid (ABA), exposure to cold, heat, water, and osmotic stress at sub-lethal levels or increasing the sucrose content in the medium can induce desiccation tolerance (Anandarajah and McKersie 1990; Senaratna et al. 1989, 1990). Another difference between the zygotic and somatic embryo is that the somatic embryo is naked and does not have a seed coat. In contrast, the zygotic embryo is encased in a seed coat. In addition to providing protection, the seed coat has other regulatory functions such as the control of gas exchange and embryo development (Gray and Purohit 1991; Senaratna 1992).

Although the zygotic embryo acquires germinability long before the seed reaches final maturity, it will not germinate unless the embryo is removed from the seed or until it is fully mature (Dasgupta and Bewley 1982; Kermode and Bewley 1985; Kermode et al. 1989). However, the relationship between the absence of the seed coat and desiccation tolerance or the control of germination of the somatic embryo has not been

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investigated thus far. Since the somatic embryo is devoid of both seed coat and desiccation tolerance, it seemed logical to investigate whether the presence of the natural seed coat influences the desiccation tolerance of somatic embryos. In this report we provide evidence that the seed coat prevents precocious germination and possesses the ability to induce desiccation tolerance in somatic embryos.

Materials and methods

Medicago sativa L. cv. Rangelander line RL34 plants were propagated by cuttings and grown in a growth chamber at 22°C and 16 h photoperiod, at a light intensity of 350 μmoles $m^{-2}s^{-1}$ provided by cool white 215 W fluorescence bulbs and 40 W incandescent bulbs. Plants were fertilized with 1/5 concentration of Hoagland solution every fourth day. Flowers were hand pollinated and tagged at two-day-intervals.

Seed desiccation

To evaluate the desiccation tolerance, seeds were harvested at different days after pollination and dried for 24 h in a desiccator (containing a saturated solution of $K_2CO_3.2H_2O$) at 43% relative humidity and incubated in the air to achieve a final moisture of ca. 10%. Dried seeds were rehydrated on filter papers (Whatman No. 9) saturated with H_20, and seeds with emerged radicals were counted to assess survival after desiccation.

Somatic embryo production

Somatic embryos of alfalfa cultivar Rangelander (Line RL 34) were produced according to the procedure described previously (Senaratna et al. 1989; 1990). Callus formation was induced from the petiole sections cultured on modified B_5 medium containing 1 mg. L^{-1} 2,4-D and 0.2 mg. L^{-1} kinetin (Astanasov and Brown 1984). The calli were then transferred into a liquid B_5 medium containing 1 mg. L^{-1} 2,4-D for cell multiplication. A cell fraction enriched with embryogenic cells was isolated by sieving the suspension sequentially through 500 μm and 200 μm nylon meshes (Nitex, B&H Thomson Ltd., Mississauga, Ontario), and plated on growth regulator-free Boi2y medium (Bingham et al. 1975) for somatic embryo development. The temperature throughout the culture process was 24 $\pm$ 1 °C and the light intensity was 75 μmol m^{-2} s^{-1} provided by cool white 215 W fluorescence bulbs with a 16-h photoperiod.

Seed coat treatment of somatic embryos

The pods were harvested at specific dates after pollination, surface sterilized with 5% calcium hypochlorite for 5 min and thoroughly washed with sterile water, and the seed coat was carefully removed from the seed aseptically. The 14-day-old cotyledonary stage somatic embryos grown in cell culture were placed inside the seed coats (one embryo per seed coat) and cultured in 9 cm Petri plates containing the Boi2y medium supplemented with 3% sucrose at the above mentioned environmental conditions. Each Petri plate contained 10 embryos and 5 plates (50 embryos) were considered as one experimental unit. Ten days after the seed coat treatment, the embryos were assessed visually for germination (elongation of root and/or shoot), and dried in a chamber with 43% relative humidity (in a desiccator containing saturated $K_2CO_3.2H_2O$ solution) and stored at room temperature and humidity for another 3 days. Dry embryos were reimbibed on filter papers saturated with sterile H_2O, in Petri plates. The embryos which became green and developed a shoot or root were counted 7 days after rehydration to assess survival. The experiment was repeated 4 times.

Extraction of seed coat material

Seed coats were removed and ground in liquid nitrogen using a mortar and pestle. The resultant powder was homogenized in distilled H_2O and centrifuged at 4000 g for 10 min. The pellet was re-extracted sequentially in methanol:water (80:20, v/v) and acetone. The supernatant was mixed and centrifuged to remove the precipitated protein. The liquid fraction was freeze-dried in the dark and dissolved in a small volume (250 μl) of 70% ethanol. This fraction was dispensed onto sterile filter paper discs (5 μl per disc) cut to the approximate size of the seed coat. The number of filter paper discs corresponded to the number of seed coats (50) used for extraction. The filter paper discs were air dried in a Laminar flow hood in the dark and placed on Boi2y medium in 9 cm Petri plates. After 1 h, somatic embryos were placed on the paper discs, the plates sealed with Parafilm, and incubated for 10 days. Somatic embryos were then removed from the discs, desiccated, and germinated under the same conditions as in the seed coat experiment.

ABA assay

Abscisic acid was extracted from the seed coats and somatic embryos using 80% methanol containing 0.1% butylated hydroxy toluene (Sigma) according to the procedure of Raikhel et al. (1987). Seed coats were ground with the extraction solution and centrifuged at 10,000 g for 20 min. The pellet was re-extracted using the same solution. The supernatant was lyophilized and dissolved in 25 mM Tris-HCl, 100 mM NaCl, 1 μM $MgCl_2$ at pH 8.5. Abscisic acid was quantified by an enzyme-linked immuno assay according to the suppliers instructions (Phytodetek-ABA, Idetek, San Bruno, California).

Osmotic potential measurements

For osmotic potential measurements, the filter paper discs containing the extract of (16, 24, and 28 DAP) seed coats (extracted and dispensed as described above) were incubated on the surface of Boi2y solid agar medium for 1 h prior to measurements. The moistened filter paper discs were removed and placed in a chamber of a sealed thermocouple psychrometer connected to a microvolt meter (Dixon Instrument Co., Guelph, Ontario). The psychrometer was placed in a thermostatic chamber at 25°C and allowed to equilibrate for 1.5 h. The osmotic potential measurement was performed after cooling for 15 sec as described by Xu et al. (1990).

Results and discussion

Growth parameters indicated that alfalfa seeds achieved the maximum fresh and dry weight at 26 days after pollination (DAP). Moisture content declined after 22 DAP and the decline continued until 32 DAP (data not shown). Germination tests of the desiccated seeds revealed that the seeds dried at 18 DAP failed to germinate (Fig. 1), and the acquisition of desiccation tolerance began at 20 DAP, reaching the maximum by 24 DAP (Fig. 1).

The tissue culture system for inducing somatic embryogenesis in this study has previously been described (Senaratna et al. 1989; 1990). The embryos could be visually detected approximately 5 days after the cell fractions enriched with embryogenic cells were plated on the growth regulator-free Boi2y medium (Bingham et al. 1975). The development of somatic

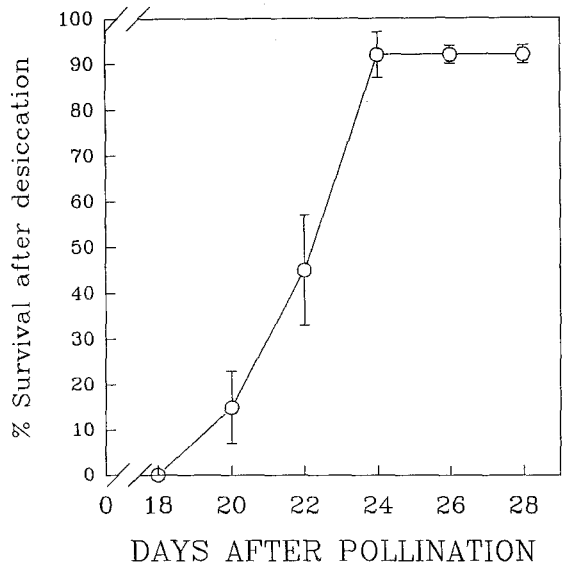

Figure 1. Survival of alfalfa seeds desiccated at different days after pollination (DAP). Each data point is the mean of four experiments; error bars, SE; error bars smaller than symbol size are contained within.

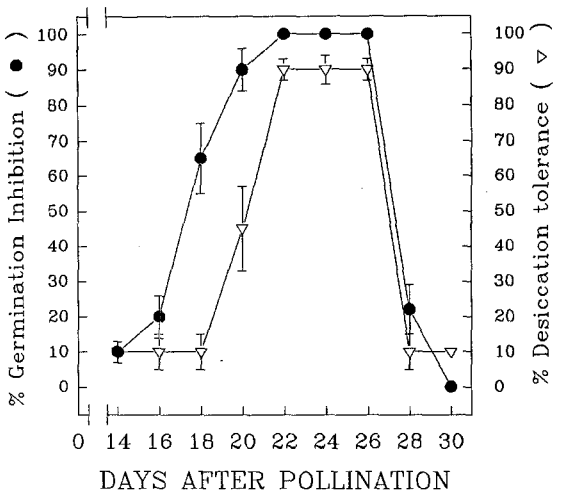

Figure 2. Effect of the alfalfa seed coats of various ages (DAP) on precocious germination and survival after desiccation of alfalfa somatic embryos. Each data point is the mean of four experiments; error bars, SE; error bars smaller than symbol size are contained within.

embryos proceeded through the globular, heart and torpedo stages reaching the cotyledonary stage by about 14 days. Alfalfa somatic embryos do not have a quiescent phase and germinate after the cotyledonary stage to produce plantlets. However, it has been demonstrated previously that desiccation tolerance can be induced in alfalfa somatic embryos by an external stimulus. The acquisition of desiccation tolerance in response to an external signal is dependant on the developmental stage, cotyledonary stage embryos being the most responsive (Senaratna et al. 1989; 1990). Thus, somatic embryos at the cotyledonary stage were placed in contact with seed coats isolated at different DAP to determine the effect of the seed coat on the induction of desiccation tolerance. When somatic embryos were in contact with the seed coats aged between 16 and 28 DAP, germination was inhibited (Fig. 2). The untreated somatic embryos germinated and developed into plantlets. The survival of somatic embryos after desiccation (ca. 15% moisture) did not necessarily correspond to the degree of germination inhibition prior to desiccation (Fig. 2). Desiccation tolerance was induced by the seed coat aged between 20 and 26 DAP, with the highest degree of tolerance by the seed coat at 22-26 DAP. None of the untreated somatic embryos germinated after desiccation. With the 20-26 DAP seed coat, the colour of somatic embryos changed from green to yellowish-white. Similar developmental changes such as inhibition of germination, degradation of chlorophyll and the acquisition of desiccation tolerance have been observed in alfalfa somatic embryos treated with exogenous ABA (Senaratna et al. 1989; 1990).

There was a discrepancy between the age at which the seed coat induced desiccation tolerance and the desiccation tolerance of the intact seed occur. This is likely a result of continual seed coat development during the treatment in vitro, despite removal from the seed. The intact seeds were desiccated at specific DAP. For in vitro treatment seed coats were removed from non desiccated seeds at specified DAP and cultured with somatic embryos, which allowed further seed coat development.

Extraction of the seed coat was performed to investigate the hypothesis that the observed effect of the seed coat was caused by a substance in the seed coat. The extract was dispensed onto filter paper discs to simulate the chemical environment of the seed coat. The germination was inhibited by the extract from the seed coats aged between 18-26 DAP. Desiccation tolerance was induced only by extracts from seed coats aged 22-26 DAP (Fig. 3). The above observations suggest that the active component or components inducing germination inhibition and desiccation tolerance is a substance that is extractable from the seed coat. Furthermore, the discrepancy between the age at which the germination inhibition and induction of desiccation tolerance occur suggests a qualitative and/or quantitative change in the substance or substances, depending on the age of the seed coat.

Abscisic acid is known to cause both inhibition of germination and desiccation tolerance in somatic embryos. At 10 μM or higher concentration, ABA applied exogenously induced complete desiccation tolerance in alfalfa somatic embryos as well as in a

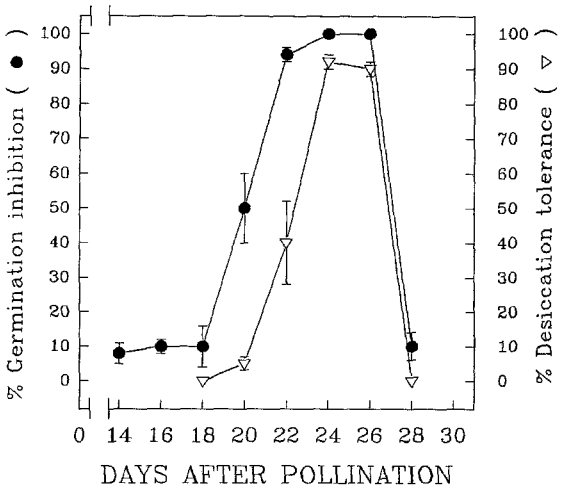

Figure 3. Effect of the extract from seed coats of various ages (DAP) on precocious germination and survival after desiccation of alfalfa somatic embryos. Each data point is the mean of four experiments; error bars, SE; error bars smaller than symbol size are contained within.

number of other species (Senaratna et al. 1990; 1991; Marsolais et al. 1991; Senaratna 1992). However, the application of 1 μM ABA inhibited germination but did not induce desiccation tolerance in alfalfa somatic embryos. Therefore, it was hypothesized that the observed influence of the seed coat is by ABA. The level of ABA in the seed coat increased from 16 DAP until 26 DAP with a subsequent sharp decline. The increased level of ABA in seeds has been observed at maturity (Finkelstein et al. 1985; Xu et al. 1990). There was a temporal relationship between the highest level of ABA in the seed coat and the highest degree of desiccation tolerance (Figs. 3 & 4). At 20 DAP, ABA content was relatively low but the inhibition of germination was high.

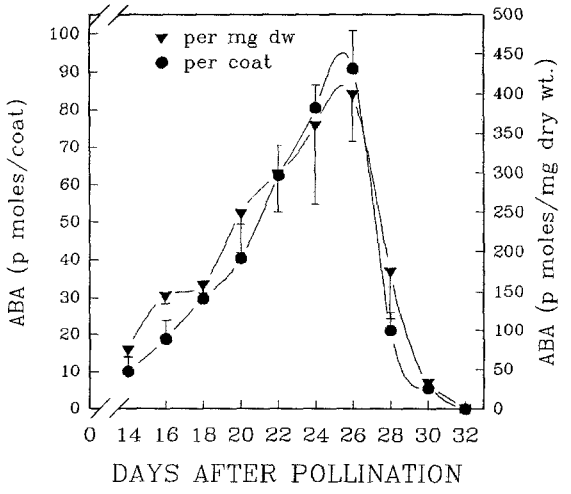

Figure 4. ABA content of alfalfa seed coats of various ages (DAP). Each data point is the mean of three experiments; error bars, SE; error bars smaller than symbol size are contained within.

These data, taken collectively, can be interpreted as the inhibition of germination occurring at both low and high ABA levels in the seed coat. Desiccation tolerance is induced only by relatively higher levels of ABA. Apparently, amounts above the critical level of ABA required for the expression of desiccation tolerance are synthesized after 22 DAP. At complete seed maturity (after 26 DAP), ABA in the seed coat is lost and the seed coat is not effective in inhibiting germination or inducing desiccation tolerance. However, this study does not rule out the involvement of substances other than ABA in the induction of desiccation tolerance.

The ABA levels within the somatic embryos placed on filter paper discs containing the seed coat extract were also measured to clarify whether ABA had migrated into the embryos. The ABA content of the somatic embryos treated with 24 DAP seed coat extract increased gradually, peaking on the third day and subsequently declining to original levels (Fig. 5), a pattern similar to that previously reported for somatic embryos treated with ABA (McKersie et al. 1990; Senaratna 1992). The extract of 16 DAP or 28 DAP seed coat did not raise the ABA content in somatic embryos (Fig. 5).

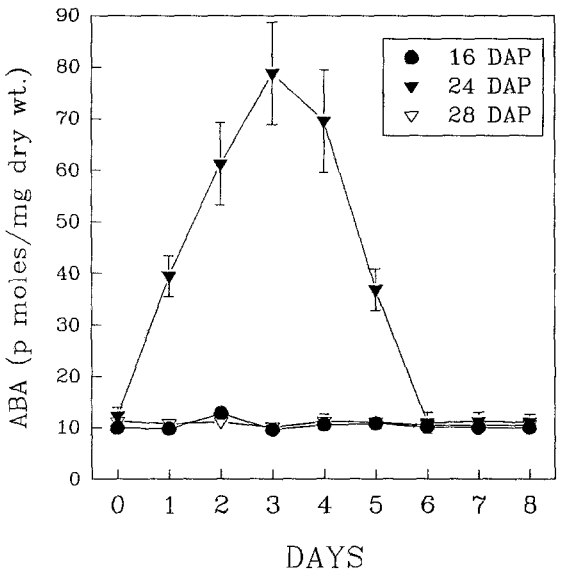

Figure 5. ABA content of alfalfa somatic embryos treated with extract from seed coat aged 16, 24 and 28 days after pollination. Each data point is the mean of three experiments; error bars, SE; error bars smaller than symbol size are contained within.

Osmotic stress has been shown to inhibit germination (Xu et al. 1990) and to induce desiccation tolerance in somatic embryos of alfalfa (Senaratna et al. 1989). Therefore the observed effect of the seed coat extract can be a result of changing osmotic potential. The osmotic potential of the filter paper discs containing 16 DAP, 24 DAP and 28 DAP seed coat extract was measured to ascertain the involvement of osmotic potential. The paper discs containing seed coat extract

were moistened on the medium for 1 h prior to the measurement to simulate the same condition during the previous experiment in which the embryos were placed on the filter paper discs. There was no significant difference in the osmotic potentials, suggesting that in this instance the germination inhibition and desiccation tolerance are not caused by osmotic changes in the surrounding environment.

Based on above experimental evidence, it can be concluded that the seed coats at certain stages of development induce desiccation tolerance and germination inhibition in somatic embryos. The absence of the seed coat around somatic embryos may be a major reason for precocious germination, lack of a quiescent phase and desiccation tolerance in somatic embryos of alfalfa.

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