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MATURATION AND GREENHOUSE PLANTING OF ALFALFA ARTIFICIAL SEEDS*

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SUMMARY

Conversion (plant production) was obtained from direct-planting alfalfa somatic embryos and encapsulated somatic embryos (artificial seeds) of alfalfa into a growth chamber and greenhouse. The embryos were planted in a commercial soil potting mix under nonsterile conditions in a manner similar to zygotic seed. Embryo maturation with abscisic acid (ABA), prior to planting, gave 48% conversion in soil under growth chamber conditions. Under greenhouse conditions, 64% conversion was obtained when humidified air was used to prevent soil surface drying. Previously, conversion in soil was between 0-6% without the ABA maturation treatment. The replacement of ABA with mannitol or combinations of mannitol and ABA during maturation resulted in lower conversion in the growth chamber than with ABA alone. ABA may be promoting the accumulation of embryo storage reserves such as proteins and carbohydrates for growth after planting in the soil environment.

Key words: alfalfa; medicago; synthetic seeds; encapsulation; somatic embryos.

INTRODUCTION

Coated somatic embryos or artificial seeds provide a potential method to deliver plant material produced from tissue culture directly to the greenhouse or field. The direct planting of somatic embryos into soil will bypass *in vitro* plant production steps which require high labor effort and expense.

Our research on artificial seeds initially focused on optimizing somatic embryogenesis to achieve high *in vitro* conversion (plant formation) from somatic embryos. That initial milestone was achieved for alfalfa (*Medicago sativa* L. var. Regen S, clone RA_3) with an *in vitro* conversion frequency of randomly-selected embryos ranging from 50-60% of all embryos (1). For our investigations in this report, we used select embryos (enlarged, well-formed embryos with distinct cotyledons and root apex) which routinely converted *in vitro* at frequencies of 70-90%. Artificial seeds were formed by coating the somatic embryos with a rigid, non-toxic alginate gel that provides protection for the embryos during the planting procedure (2).

A major obstacle in the use of artificial seeds as a plant propagation system has been insufficient somatic embryo quality and vigor for direct planting outside an *in vitro* environment. Much of the artificial seed research has focused on somatic embryogenesis and not on the later stages of the system such as conversion and sowing under natural soil conditions. Investigations into desiccated artificial seed systems have been reported by Kitto and Janick (3,4) and Gray (5,6), but planting into a soil environment was not attempted. Baker (7) investigated adding sucrose, plant hormones, and other nutrients to a gel for fluid drilling of carrot somatic embryos in a greenhouse, but embryo survival was low and no plants were produced.

We previously reported planting alfalfa artificial seeds in a non-sterile soil potting mix in a growth chamber and obtained a high of 18% conversion on one occasion (8). Most often, however, we obtained conversion frequencies of less then 6%. In this report we present results of improved conversion of artificial seeds planted in a soil environment under growth chamber and greenhouse conditions.

MATERIALS AND METHODS

Alfalfa has a well-developed, stage-specific somatic embryogenesis system (1,9). Callus was produced from leaf petioles placed on Schenk and Hildebrandt medium (SH) (10) supplemented with 25 μM naphthaleneacetic acid and 10 μM kinetin (11). The callus was placed for 3 weeks on SH with potassium nitrate replaced by 20 mM potassium citrate and 25 mM glutamine. The callus was induced to form embryos on SH medium with 50 μM 2,4-dichlorophenoxyacetic acid and 5 μM kinetin for 3 days (12). The induced callus was placed on SH medium with 10 mM ammonium nitrate and 30 mM proline causing numerous somatic embryos to form after three weeks at 26° C (9).

Soil planting techniques were developed that were amenable for high survival and growth of somatic embryos. Embryo survival and conversion occurred when the embryos were planted on the surface of pasteurized greenhouse soil potting mix (10 peat: 9 vermiculite: 1 perlite, McCalif Growers Supplies, San Jose, CA). The loose structure of the mix allowed for good root penetration from the somatic embryos into the soil. The embryos were placed on the soil surface in shallow furrows, cotyledons facing upright, and left without any soil covering. The high humidity in the growth chamber deterred soil drying especially on the surface of the potting mix

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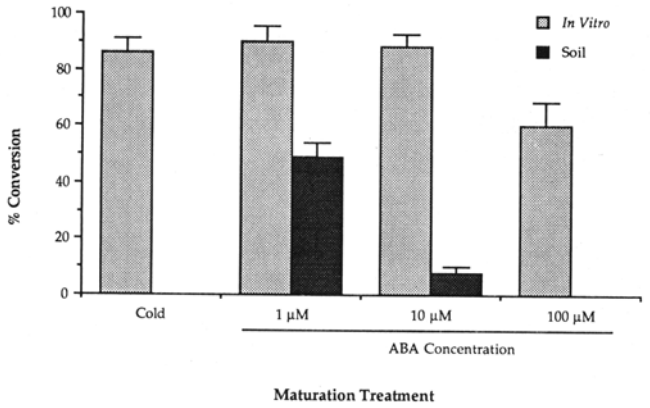

FIG. 1. Effect of ABA maturation on alfalfa somatic embryo conversion in soil in a growth chamber. Conversion was scored 4 weeks after sowing. Bars represent one standard error.

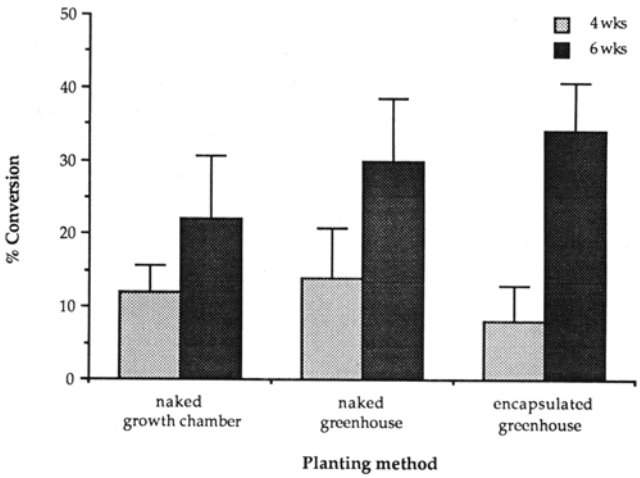

FIG. 2. Conversion of naked (not encapsulated) or encapsulated alfalfa somatic embryos (artificial seeds) in soil in the greenhouse and growth chamber. Conversion was scored 4 and 6 weeks after sowing. Bars represent one standard error.

where the embryos were planted. The growth chamber was maintained at 24° C with 16 hours of fluorescent light per day. All treatments contained 5 replicates of 10 embryos unless indicated.

In the ABA maturation experiments, selected embryos were placed onto SH containing varying levels of ABA for 14 days at 24° C in the dark, or embryos were left on the original regeneration medium and stored at 4° C in the dark ("cold" treatment). The embryos were planted in soil and converted in a growth chamber or planted *in vitro* on half-strength SH, with 1.5% maltose instead of sucrose, and 25 μM GA3. *In vitro* conversion was at 24° C under 16 hours of fluorescent light per day.

In the ABA + mannitol maturation experiments, somatic embryos were placed onto SH containing various concentrations of ABA and mannitol for 16 days at 24° C in the dark. The embryos were planted in soil potting mix and converted in a growth chamber.

In the greenhouse experiments, somatic embryos were matured on SH containing 5 μM ABA for 21-27 days in the dark. Somatic embryos and artificial seeds were hand-planted into styrofoam transplant trays, filled with potting mix, with one embryo or artificial seed per cell. The trays were placed into a greenhouse and watered regularly by hand with a spray bottle. A second set of somatic embryos were planted in the greenhouse in transplant trays and either watered by hand, misted using overhead sprinklers, or humidified in a tented area during conversion. Encapsulation was done by inserting individual embryos into drops of 2% LF60 alginate (Protan Scotia Marine, Norway) complexed in 100 mM calcium nitrate for 20 minutes followed by a rinse in water.

TABLE 1

EFFECT OF ABA AND MANNITOL ON CONVERSION OF SOMATIC EMBRYOS PLANTED IN SOIL IN A GROWTH CHAMBER[a]

ABA Concentration (μM)	Mannitol Conc. (%)	Conversion (%)	
		Average	Std. Error
10	0	32.0	9.2
10	8	4.0	2.5
1	8	10.0	4.5
0.1	8	14.0	5.1
0	8	16.0	4.0

[a]Conversion was scored 6 weeks after sowing.

Somatic embryos were scored for embryo to plant conversion using rigid evaluation criteria. We previously defined an *in vitro*-converted alfalfa plant as possessing a shoot (with two or more trifoliate leaves), branched roots, little swelling or callusing of the shoot/root connection, and a normal alfalfa phenotype (2). Likewise, we imposed rigid evaluation criteria on the plants that were produced in the soil environment. The definition of a plant produced in soil was the presence of two or more trifoliate leaves, a root that extended and anchored into the soil structure, and a normal alfalfa phenotype without stunting or leaf chlorosis. These evaluation criteria assured that conversion scores reflected only the number of high-quality, vigorous plants produced from somatic embryos.

RESULTS AND DISCUSSION

Somatic embryo germination was arrested during exposure to the ABA maturation media. The ABA and dark incubation conditions caused the embryos to turn from green to white and produce enlarged cotyledons. At concentrations less than 1 μM ABA, the embryos were not arrested and germinated readily on the ABA medium. Therefore, ABA concentrations less than 1 μM were not used in the experiments. After ABA maturation, the embryos were planted in non-sterile potting soil for conversion of the embryos in soil (Figure 1). The highest soil conversion of 48% was obtained when the embryos were first matured on medium containing 1 μM ABA. Embryos matured on 10 μM ABA converted at a lower frequency while the highest level of ABA tested (100 μM) and cold-treated embryos did not convert at all under soil conditions. We have subsequently used the ABA concentrations of 1-5 μM for maturation of hundreds of somatic embryos and have routinely obtained conversion frequencies of 30-60% in soil in growth chambers. In contrast, we previously obtained less than 6% conversion in soil from embryos prior to development of the ABA maturation treatment.

Under *in vitro* conversion conditions, all treatments had similar high conversion frequencies in the growth chamber, with a slightly lower frequency for the SH + 100 μM ABA treatment (Figure 1). All treatments converted at a higher frequency *in vitro* compared to soil conditions. This was probably due to the presence of sucrose, salts, and other growth nutrients in the *in vitro* medium. The increased conversion frequency of somatic embryos in soil due to ABA

maturation, coupled with high *in vitro* conversion frequencies, implies that ABA may be promoting the storage of carbohydrates, proteins, and other nutrients within the embryo with subsequent radicle and shoot growth in the nutrient-poor soil environments. ABA is known to accumulate in developing zygotic embryos and exogenously applied ABA has an important role in controlling embryo germination (13,14,15,16). Somatic embryos may also require ABA for continued biochemical and physiological development prior to conversion. While somatic embryo conversion frequency in soil was improved by ABA, the conversion process proceeded at a slower rate than that of zygotic seed. In somatic embryos,the first true leaf appeared approximately two weeks after planting whereas in zygotic seed the first leaf appears after approximately one week. While ABA maturation significantly improved the conversion frequency and vigor of somatic embryos planted in soil, other developmental factors are needed to increase the overall conversion rate to coincide with that of zygotic seed.

We tested the effect of osmotic treatment on somatic embryos to determine if we could improve soil conversion in a manner similar to ABA. An osmoticum, like ABA, can inhibit precocious germination of embryos *in vitro* (17). We determined that the minimum level of an osmoticum, mannitol, to arrest germination of the somatic embryos was 8% (unpublished). Somatic embryos were matured on ABA, 8% mannitol, or combinations of ABA + 8% mannitol and then converted in soil. Low ABA concentrations could be tested only in combinations with mannitol since the somatic embryos germinated rapidly on the maturation medium containing low ABA. Embryos matured on mannitol or ABA + mannitol combinations converted in soil in the growth chamber but at a lower frequency than embryos matured on ABA alone (Table 1). The osmoticum arrested the germination of the somatic embryos during maturation but did not duplicate the high conversion frequencies seen with ABA. Immature zygotic embryos of wheat and barley cultured on ABA or mannitol were observed to have similar changes in morphology, fresh weight, protein, and lectin content (18). However, while the endogenous ABA content increased in mannitol-cultured barley embryos, wheat embryos showed no change (19). Rape zygotic embryos cultured on sorbitol also did not exhibit elevated ABA levels compared to embryos cultured without sorbitol (20). The results suggest that osmotic stress and ABA provide separate physiological signals for embryo maturation.

Ideally, artificial seeds would be used for planting clonal material directly into the field. Until somatic embryo quality (vigor and conversion frequency) reaches that of zygotic seed, an intermediary use for artificial seeds is the production of field-ready transplants in the greenhouse. Automated sowing of artificial seeds should be possible using modified vacuum seeders to singulate the artificial seeds into transplant trays. We investigated sowing embryos in the greenhouse by hand-planting somatic embryos and artificial seeds in potting soil in trays. The trays were carefully watered with a spray bottle. Conversion frequencies were 30% for naked embryos and 34% for artificial seeds when measured six weeks after planting (Figure 2). These frequencies in the greenhouse were found to be similar to those in the growth chamber. Plants produced under greenhouse conditions had vigorous growth (Figure 3). These plants were transplanted into peat pots and later into the field, with high plant survival and good stand establishment.

Various greenhouse watering methods were also investigated. The highest conversion frequency was 64%, obtained using a humidity tent watering system. Hand watering

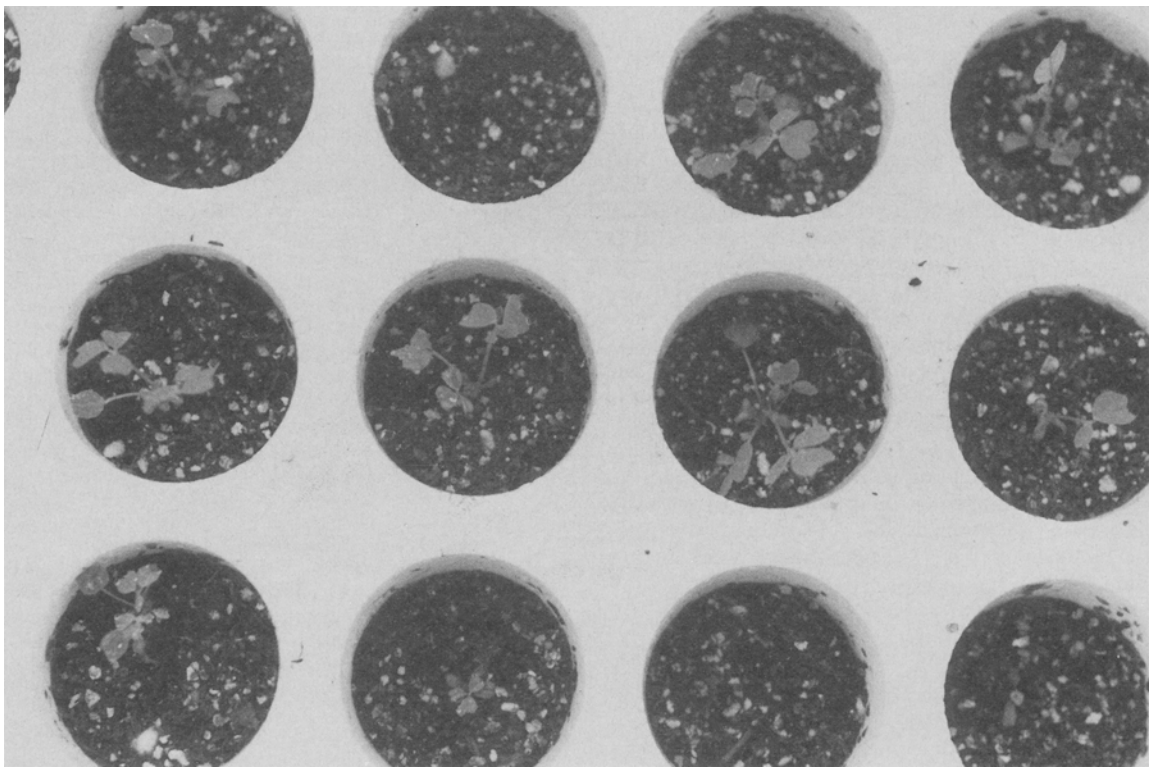

FIG. 3. Alfalfa plants produced from naked somatic embryos sown into potting mix and converted in the greenhouse. There was one embryo planted in each cell. The plants are 6 weeks old, which is the time conversion was scored.

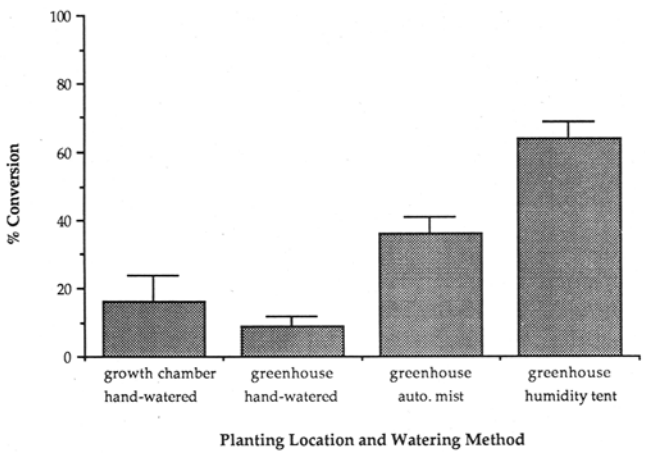

FIG. 4. Effect of various watering methods on conversion of alfalfa somatic embryos in soil in the greenhouse. Data were taken six weeks after sowing and represent averages of 5-10 replicates of 10 embryos each. Bars represent one standard error.

and watering with an overhead mist resulted in 9% and 36% conversion, respectively (Figure 4). The higher conversion under the humidity tent system may have been due to the constantly moist soil surface rather than the wet and dry cycles experienced by the hand-watered and overhead misted treatments. Thus, large-scale production of transplants using artificial seeds may be best accomplished in greenhouses where high humidity can be maintained using foggers or other types of fine misting. Conversion under the humidity tent watering system was much higher than in the controlled conditions of the growth chamber. This may be due to higher light quality and intensity in the greenhouse which would increase plant growth rate and possibly plantlet survival.

An estimation of phenotypic variation in plants produced from somatic embryos was quantitated by scoring plants for gross morphological abnormalities. Seven-month old plants produced from somatic embryos converted under greenhouse conditions were scored for aberrant leaf types (shape and color) and stand morphology (stunting and form). One hundred twenty-nine plants were evaluated and 93% were found to have a normal phenotype for the RA_3 variety. Other RA_3 plants produced from somatic embryos were grown to flowering in the greenhouse and also showed a high frequency of true-to-type flower color (purple) as well as growth rates (unpublished). Although we have just begun to assess the level of somaclonal variation in plants produced from somatic embryos, early indications are that most of the plants produced from artificial seeds have very few gross morphological abnormalities. More rigorous evaluation of these plants will be done to determine if various tissue culture conditions can aid in stabilizing the genome. We hypothesize that rigorous tissue selection during somatic embryogenesis and embryo maturation have selected against weak or abnormal plants.

CONCLUSION

The achievement of consistent conversion of somatic embryos and artificial seeds under soil conditions had been a major roadblock in establishing the feasibility of using this propagation method for mass propagation of plants. We have demonstrated that artificial seeds can serve as a direct delivery system of tissue-cultured material to a soil environment under growth chamber as well as greenhouse conditions. While artificial seeds will provide the greatest economic benefit when sown under field conditions, greenhouse transplant production may provide an intermediate method for the mass propagation of selected genotypes. Future research must continue to move away from *in vitro* experimentation and towards improving embryo vigor and growth rates under soil conditions.

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