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Regeneration of *Coronilla varia* L. (crownvetch) plants from callus culture*

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Abstract. *Coronilla varia* L. (crownvetch) plants were regenerated from callus cultures through somatic embryogenesis. Callus cultures were initiated using hypocotyls excised from sterile seedlings. Cultures were then transferred from a modified Gamborg's B5 medium containing 2,4-D to a medium containing no plant growth regulators (basal B5). Formation of embryos was evident in 12 of 32 callus lines after transfer of callus to BOi2Y (modified Blaydes' medium supplemented with 100 mg inositol and 2 g yeast extract/L). Basal B5 supplemented with 10 *mM* asparagine or 20 *mM* NH_4Cl could be substituted for BOi2Y. Embryos subsequently transferred to basal B5 developed roots and shoots. Plants thus formed were first transferred to vermiculite and then to soil.

Introduction

Regeneration of plants from cell and callus culture is not only of interest as it relates to physiological and morphological processes, but also has utility in breeding programs [1]. Practical applications of developing techniques such as protoplast fusion, improvement of plants by selection at the cellular level, and modification of plant genomes depend on recovery of plants from selected or modified cells [14]. Among forage legumes, plants have been regenerated from alfalfa and white clover protoplasts [3, 5] and from suspension cultures of red clover [9], berseem clover [8], and birdsfoot trefoil [12]. Regeneration of plants from callus has been achieved in three other forage legume species [7].

Coronilla varia (crownvetch) is widely planted in the northeastern United States for erosion control but has limited use as a forage crop. Problems associated with use of this legume include lack of persistence under continuous grazing, low palatability, and the presence of several 3-nitropropanoyl-D-glucopyranose esters. These aliphatic nitro compounds are toxic to nonruminants upon ingestion but are degraded by rumen microflora and thus do not affect ruminants [4, 6].

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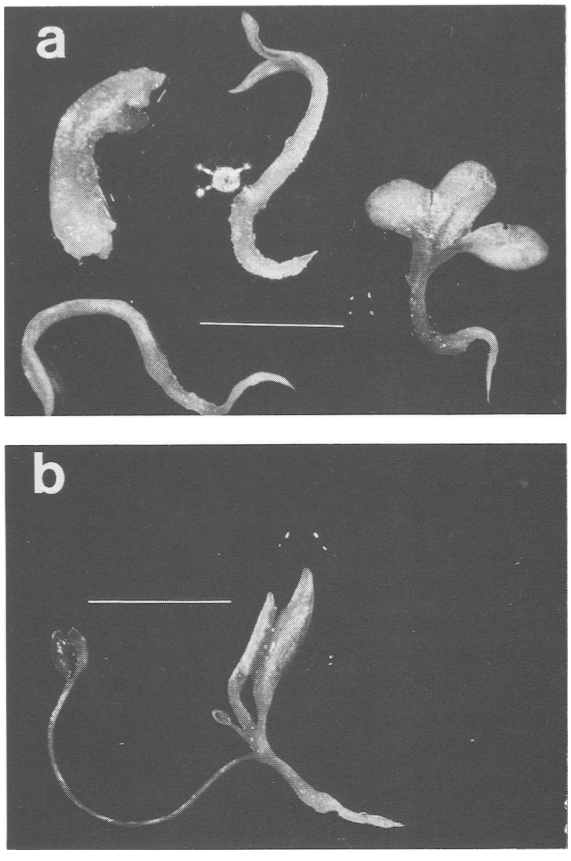

Figure 1. Stages in production of plants from embryos: a) embryos formed from tissue grown 4 weeks on BOi2Y; b) embryo with shoot developing at cotyledonary node. (Bars equal 1 cm).

Since employment of in vitro techniques in crownvetch improvement requires recovery of plants from culture, we undertook a study of in vitro culture and regeneration of *C. varia*. This paper describes the establishment of callus cultures from *C. varia* seedlings and mature leaf tissue. Regeneration of plants through somatic embryogenesis in hypocotyl-derived callus cultures is reported.

Materials and methods

Hypocotyls from sterile seedlings of *C. varia* L. cv. Chemung were used to initiate callus cultures. Seeds were surface-sterilized in 1.05% sodium hypochlorite under partial vacuum for 15 min, rinsed 3 times with sterile distilled water (SDW), and germinated in 25 ml SDW in foil-covered 125 ml Erlenmeyer flasks on a rotary shaker. Hypocotyls excised from 1.5- to

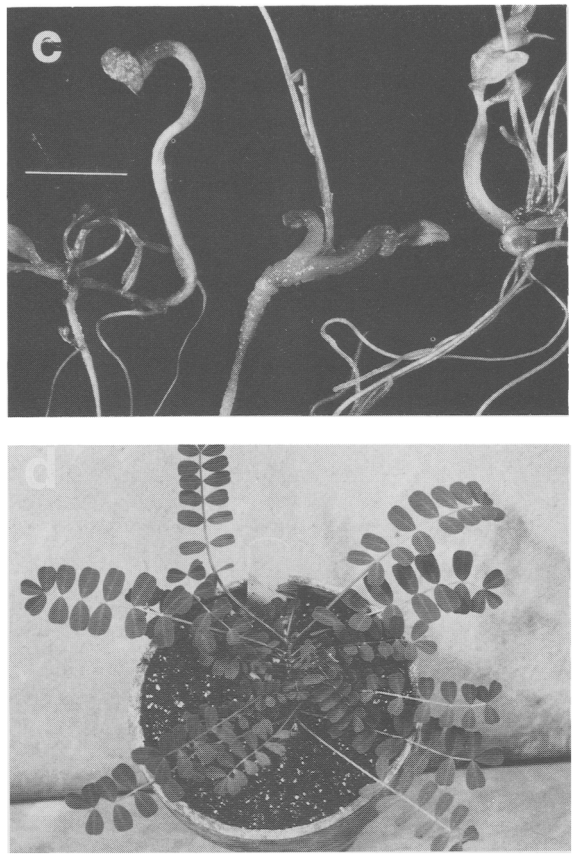

Figure 1. c) shoot development in irregular embryos; d) plant obtained through somatic embryogenesis. (Bars equal 1 cm).

2.0-cm-long seedlings were transferred to Gamborg's B5 medium [2] modified to contain 5.66 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 1.34 μM NAA (naphthaleneacetic acid), and 1.16 μM kinetin (G2 medium). All media were solidified with 0.8% agar. Calli originating from different hypocotyls were maintained as separate lines. Transfers were made to fresh medium at 4-week intervals. Areas of callus producing chlorophyll or showing signs of differentiation were selected for transfers because embryogenesis occurred at these sites. Cultures were grown on 33 ml of medium in foil-covered 125 ml Erlenmeyer flasks and were maintained at 25 °C under cool-white fluorescent lights (10 $\mu E\, m^{-2}\, s^{-1}$) with a 16-hr photoperiod.

After establishment of cultures from hypocotyls (2-4 transfers at 4-week intervals), tissue was transferred sequentially from G2 to D2OK2 medium (Gamborg's B5 medium modified to contain 20 μM 2,4-D and 2 μM kinetin), basal B5 (Gamborg's B5 without plant growth regulators), and BOi2Y

(modified Blaydes' medium supplemented with 100 mg inositol and 2 g yeast extract/L [11]). Embryos formed on BOi2Y were transferred to basal B5 and, after formation of roots and shoots, potted in vermiculite. To prevent dessication, these plants were covered with beakers for one week after transfer to vermiculite and watered as needed with one-half strength Hoagland's solution. After 4 to 6 weeks, plants were potted in soil.

Results and discussion

Callus cultures could be readily initiated from excised hypocotyls of *C. varia*; 45% of explanted hypocotyls produced callus that could be maintained by serial transfer and yielded 32 separate callus lines. The remainder produced no callus or callus that did not grow upon serial transfer.

Embryo formation was apparent in 12 of 32 callus culture lines after sequential transfer at 4-week intervals from G2 to D2OK2, basal B5, and BOi2Y. The 4-week interval on a 2,4-D-free medium between D2OK2 and BOi2Y was necessary for production of large embryos capable of developing into plants. Tissue grown on B5 with 2,4-D (or 2,4,5-T) at 10 or 20 μM, upon transfer to BOi2Y, formed small embryos incapable of further development. Addition of NAA, indolebutyric acid, p-chlorophenoxyisobutyric acid, or kinetin at 10 or 20 μM to B5 basal medium neither promoted nor inhibited subsequent embryo production on BOi2Y.

The tissue in one flask of BOi2Y medium (4 callus pieces and 5–10 g per flask after 4 weeks) was sufficient for demonstration of embryogenesis in a line. Embryo production varied from a few embryos per flask to differentiation of entire callus pieces into embryos of various sizes.

The bulk of embryo development occurred after transfer of tissue to BOi2Y, but the initial stages of embryogenesis may well have taken place earlier. In callus lines with the highest embryogenic potential, small embryos were formed by callus grown on basal B5, but these did not enlarge and develop further until transfer to BOi2Y. BOi2Y could be replaced with basal B5 supplemented with 1.0 mM asparagine or 20 mM NH_4Cl. This suggests that the level of reduced nitrogen in basal B5 (2 mM), while adequate to support 2,4-D-promoted growth on D2OK2, was insufficient for embryo enlargement and development.

Embryos formed by callus growing on BOi2Y were variable in size and morphology (Figure 1a, b, c). Some had two cotyledons and areas analogous to a hypocotyl and radicle. Embryos with single or multiple cotyledons were common (Figure 1a). Embryos rooted and formed shoots either on BOi2Y, or more frequently, upon subsequent transfer to basal B5. Irregular embryos with no axes of symmetry ultimately rooted at several points. In embryos with two or more cotyledons, shoots originated from the cotyledonary node (Figure 1b). Embryos with a single cotyledon developed shoots from the hypocotyl or roots (Figure 1c).

Optimum conditions for transfer of plantlets from flasks to soil were not determined. The majority of plantlets failed to survive this transition, but the 29 plants successfully transferred to soil appeared phenotypically normal (Figure 1d). Sixteen of the established plants, all regenerated from one callus line, were transplanted to the field in 1982; all plants flowered during the succeeding year.

Leaves both from plants grown from seed and plants regenerated from one callus line were used to establish callus cultures. Eight mm discs were cut from leaves surface-sterilized as previously described [7] and transferred to G2 medium. All discs produced callus cultures; however, embryogenesis could not be induced in these cultures by using the protocol developed for hypocotyl-derived callus cultures.

In vitro regeneration of plants occurs either by direct formation of roots and shoots or through somatic embryogenesis with subsequent formation of roots and shoots from embryos [10]. Although direct formation of roots and shoots is the more common route, embryo formation has been reported in a limited number of species [13]. Of the eight forage legumes that have been regenerated in vitro, somatic embryogenesis has been reported only in alfalfa [15] and red clover [9]. This report adds *C. varia* to the list of species capable of in vitro regeneration through somatic embryogenesis.

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