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# Supernodulation and Non-Nodulation Phenotypes of *Glycine max* (Soybean) are Stable through Organogenic and Embryogenic Regeneration

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**Abstract**

Conditions for the regeneration of soybean *Glycine max* cultivar Bragg and its derived nodulation mutants nts382, nod49, and nod139 (Carroll et al., 1985a; b; 1986) via organogenesis and embryogenesis were investigated. The *nts* locus conditions abundant nodulation and tolerance in nodulation to otherwise inhibitory levels of nitrate. The nod49 mutant (allelic to *rj1*) governs non-nodulation, as does nod139 (at the *rj6* locus). All genes are unlinked. High benzylamino purine (BAP) levels (50–100 μM) were optimal for shoot organogenesis from cotyledonary petioles of Bragg and the nodulation mutants. Embryogenic liquid cultures were initiated of cv. Bragg and the nodulation mutants nts382, nts1007 nod49 and nod139. Plants from all genotypes were regenerated from the cotyledonary petiole organogenic system while only plants of nts1007 were regenerated from the embryogenic suspension culture system. The nts1007 plants regenerated from embryogenic suspension cultures and the plants from shoot organogenesis of the cotyledonary petiole were fertile and showed the same nodulation phenotypes as the parental material. The described protocols and cultures may be useful for gene transfer into these soybean genotypes.

Keywords: nodulation, tissue culture, nitrogen fixation, symbiosis

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## 1. Introduction

The nodulation mutants of Bragg used in this study were generated by ethyl methyl sulfonate (EMS) mutagenesis and F2 family selection (Carroll et al., 1985a; b; 1986). Non-nodulating mutants nod139 and nod49 failed to nodulate (Carroll et al., 1986; Mathews et al.; 1989a; b) whereas the nitrate tolerant symbiosis (*nts*) allelic mutants nts382 and nts1007 (Carroll et al., 1985a; b) had a supernodulating phenotype (up to 40 times the nodule number of the wild-type Bragg parent). The *nts* locus has been the focus of extensive molecular mapping work (see Gresshoff, 1993). It is located on linkage group H of the USDA/ARS RFLP map (Keim et al., 1990), being less than 1 cM from the cloned pUTG-132a marker (Landau-Ellis et al., 1991; Landau-Ellis & Gresshoff, 1992).

The non-nodulation mutants nod49 and nod139 are located in two different genes (*rj1* and *rj6*) (Mathews et al., 1989b, Pracht et al., 1993) despite their common non-nodulation phenotype. *rj1*, *rj6* and *nts* segregate independently, suggesting that they are on different chromosomes (Mathews et al., 1990; 1992).

The molecular mapping of these genes is a prerequisite for their eventual positional or map-based cloning. As part of this approach, transformation of cloned sequences of putative assignment into mutant cells or plants will help to ascertain the identity.

Regeneration of *G. max* by shoot organogenesis from cotyledonary node tissue has been demonstrated in a wide range of genotypes from all maturity groups (Cheng et al., 1980; Saka et al., 1980; Wright et al., 1986a, b). Hinchee et al. (1988) used $B_5$ based medium (Gamborg et al., 1968) supplemented with 5 μM BAP benzyl-amino purine) and cotyledonary node explants infected with *Agrobacterium tumefaciens* to regenerate transgenic plants of cultivar Peking. Wright et al. (1986b) demonstrated shoot organogenesis from cotyledonary explants in ten commercial cultivars using MS medium (Murashige and Skoog, 1962) supplemented with 5 μM BAP. These studies demonstrated that soybean cultivars differ in their ability to generate through embryogenesis or organogenesis.

In contrast to these organogenic cultures, Christianson et al. (1983) initiated regenerable embryogenic suspension cultures from immature zygotic embryo axes (cv. Mitchell). Ranch et al. (1985) also reported the production of embryogenic cultures of soybean. These embryos were initiated at a lower level of 2,4-D (2,4-dichlorophenoxy acetic acid; 10 $\mu g.ml^{-1}$) and did not produce secondary embryos.

Finer and Nagasawa (1988) initiated more usable embryogenic suspension cultures from immature cotyledons of cultivar Fayette. Embryogenic tissue capable of producing secondary embryos upon embryos was produced by culturing the immature cotyledons for several months at a very high level of 2,4-

D (40 mg/l). This tissue was then used to initiate embryogenic suspension cultures termed 'highly embryogenic ontogenetic and early staged' suspensions. Ranch et al. (1985) published the production of embryogenic cultures in soybean. These embryos were initiated at a lower level of 2,4-D (10 mg/l) and did not produce secondary embryos. Thus, the system was not suitable for the production of suspension cultures but only for the direct production of plants.

In 1991 Finer and McMullen showed the regeneration of transgenic plants from embryogenic suspension cultures. Bailey and Parrott (1992) also reported initiation of embryogenic suspension cultures and regeneration of fertile plants from cultivars Century, Davis, Lee, Hutcheson, Peking and PI 417138.

Cotyledonary petioles of cv. Peking (inoculated with *Agrobacterium tumefaciens*) were used as the regeneration system by Hinchee et al. (1988) to produce transgenic plants. Embryogenic suspension cultures have also proven to be efficient regeneration systems when used for particle gun transformation of soybean (Finer and McMullen, 1991).

It is hoped that either regeneration system will facilitate transformation systems aimed at elucidating the mutated genes responsible for the nodulation phenotypes of these soybean lines.

## 2. Materials and Methods

*Embryogenic regeneration system*

*Selection of plant tissue.* Soybean plants *G. max* (L.) Merrill, cv. Peking, cv. Bragg and its nodulation mutants nts382 (supernodulation), nod49 and nod139 (both non-nodulation) were greenhouse-grown in soil supplemented with Osmocote$^{TM}$ (14:14:14) fertilizer. The nodule phenotype was checked four weeks after sowing and inoculation with *Bradyrhizobium japonicum* strain USDA110. Between November and March plants were grown with the aid of supplemental lighting from metal halide lights (16/8 hr light /dark photo-period). Plants with immature pods of a suitable size were obtained 2–3 months after sowing. Pods (30–40 mm long) containing immature seeds approximately 4–5 mm in length were harvested. Seed size was checked by holding the pod up to the light. Only pods developed from the first flowers on each plant were used.

*Initiation of embryogenic callus.* Initiation and culture of the embryogenic suspensions were performed as described by Finer and Nagasawa (1988) with a few modifications. Immature pods were surface-sterilized for 20 minutes in a 20% solution of commercial bleach containing 0.05% Tween-20, then rinsed four times in sterile distilled water. The sterile pods were cut open aseptically and the immature seed dissected out. The cotyledons were separated and the embryo

and seed coat removed. The cotyledons were then placed abaxial side down on a medium composed of MS salts (Murashige & Skoog, 1962), Gamborg's $B_5$ vitamins (Gamborg et al., 1968), 6% sucrose, 40 mg/l 2,4-D and 0.8% purified agar (Sigma Comp. St. Louis). The pH for culture was modified from 5.6 to 7 according to Komatsuda and Ohyama (1988). All media were autoclaved for 20 min at 121°C, 15 psi. The cultures were maintained at 26°C in an illuminated incubator (40 $\mu Em^{-2}s^{-1}$, 16/8 hr light/dark photo-period). The tissue was subcultured every 4 weeks onto the same medium for a period of between 2 to 6 months until embryogenic tissue formed on the callused tissue.

*Initiation of embryogenic suspension cultures.* The embryogenic tissue formed was dissected from the explant callus and analyzed under the dissecting microscope.

If small secondary embryoids were seen initiating from the primary embryos, the tissue could be used to initiate suspension cultures. Approximately 2 mm diameter pieces of this embryogenic tissue were placed in sterile 125 ml DeLong flasks containing 35 ml of suspension medium. This medium (Finer and Nagasawa, 1988) consisted of MS salts modified by the replacement of the MS nitrogen with 10 mM $NH_4NO_3$ and 30 mM $KNO_3$, $B_5$ vitamins (Gamborg et al., 1968), 6% sucrose, 5 mg/l, 2,4-D and 5 mM asparagine (pH 5.7). Flasks were capped with stainless steel closures and sealed with Para-film™. Suspensions were then placed at 24°C on a shaker agitated at 150 rpm and continuously illuminated with a light intensity of approximately 60 $\mu Em^{-2}s^{-1}$. The suspensions were subcultured every 3 to 4 weeks at very low inoculum density (two 2 mm diameter clumps per flask). If the medium became cloudy the suspensions were subcultured early.

*Regeneration of plants from suspension cultures.* Small clumps (2 mm diameter) of green embryogenic suspension were removed from suspension and placed on a medium composed of MS salts, Gamborg's $B_5$ vitamins, 6% maltose, 0.2% Gel-rite (Phytagel, Sigma) and 0.5% activated charcoal (J. Bailey, Georgia State University, pers. comm.), pH 5.7. These embryos were incubated under the same conditions as for embryo induction. After 4–8 weeks of growth the embryos gradually turned yellow, indicating that they had reached physiological arrest and were ready for desiccation. Yellow embryos of a minimum size of 5 mm in length were desiccated in the dark at room temperature for 3–7 days. Approximately 10–15 embryos were desiccated in a wrapped (Para-film™) 15 × 100 mm Petri dish. It was important to desiccate the embryos only to a 'flaccid' and not a 'crisp' stage. After desiccation the embryos were again placed on the embryo development medium which was only modified by the addition of 3% sucrose instead of maltose. Three to four weeks were required to allow normal root and shoot development. During this stage the Petri dishes were wrapped in Scotch™ pressure sensitive tape (vent tape) and opened in the sterile laminar

flow hood every 7 days for 10 minutes. This prevented the build-up of ethylene which could arrest germination. Once a normal root system and shoot had developed the plants were transferred for two weeks to Magenta™ jars and grown in 20 ml of liquid medium consisting of Monniers salts (1976) and Morel and Wetmore vitamins (1951; Mon-Mor medium). This step stimulated rapid root and shoot growth. The resultant plantlets were transferred to 50% Vermiculite™ 50% Faffard™ soil-less compost and cultured under a plastic bag for 1–2 weeks to acclimatize the plants to greenhouse conditions. They were then inoculated with 10 ml of $10^6$ $ml^{-1}$ *B. japonicum* USDA110 both upon transfer to soil and once more 2 weeks later. The plants were checked to see if they displayed normal nodulation profiles six weeks after transfer to soil. They were then grown to maturity and checked for nodule phenotype, flower fertility, and seed set.

*Organogenic cotyledonary node regeneration system*

Seeds of cultivars Peking, Essex, Bragg and nodulation mutants nts382, nod49, nod139 were hand-harvested from greenhouse-grown plants. Their nodulation phenotypes were verified upon harvest. The seeds were surface-sterilized by the following procedure; a five minute wash in a soapy aqueous solution of the fungicides Captan™ and Banrot™, followed by a 10 min wash in 40% Chlorox, containing 0.1% Tween 20, then five rinses in sterile distilled water. Vigorous agitation was employed at each stage. The seeds were allowed to soak for one hour in an aqueous solution of Captan™ and Banrot™ before plating on a medium (1/10th $B_50$) composed of 1/10th $B_50$ salts, 20g/l sucrose, 8g/l purified agar (Sigma) at pH 5.7. $B_50$ medium was identical to 1/10th $B_50$ medium except that full strength Gamborg's $B_5$ salts were used. The culture procedure for inducing shoot organogenesis from the cotyledonary node was basically as described by Wright et al. (1986a; b) and Hinchee et al. (1988) with some media modifications. After 6–10 days of culture under continuous light (60 $\mu Em^{-2}s^{-1}$) at 27°C the green cotyledons were removed from the seedling with approximately 5 mm of the petiole. The cotyledons were separated by a longitudinal cut through the adjoining hypocotyl tissue. The seedling meristem was carefully removed and the cotyledons placed adaxial side down on a regeneration medium ($B_50$+5 μM BAP) consisting of $B_5$ salts, 20 g/l sucrose, 8 g/l purified agar (Sigma) and 5 μM BAP, pH 5.6. Further experiments were performed with BAP levels of 0 to 300 and 0 to 800 μM in the regeneration medium. Dissected cotyledons of all of the aforementioned cultivars were cultured on these media ($B_50$ + various BAP levels) for 4–8 weeks. The rate of shoot organogenesis determined the amount of time the explants were left on the media. Sub-cultures were generally performed every 4 weeks. The explants were placed on $B_50$ medium for 4 weeks for shoot

elongation, then 2 weeks on 1/10th $B_50$ medium for rooting. Shoots could be divided from the explant at this rooting stage. The resultant plantlets were transferred to Vermiculite and cultured under a clear plastic bag for 1–2 weeks to acclimatize the plants to greenhouse conditions. The regenerant plants were then either placed in plastic pouches (Vaughan's Seed Co., Downers Grove, Ill.) or in 50% Vermiculite 50% Faffard™ soil-less compost and inoculated with strain USDA110.

*Analysis of nodulation: plastic growth pouches.* One tissue culture regenerated plant and one 5 day old seedling of the same mutant genotype were placed in a plastic growth pouch, watered with one fourth strength Herridge's solution (Delves et al., 1986) and cultured for 3 days in a growth chamber. The culture conditions were 500 $\mu Em^{-2}s^{-1}$ illumination with 70% humidity and a 16:8 hr day cycle set at 25°C. On the third day the plants were inoculated with 100 µl of strain USDA110 ($10^6$ cells/ml). The plants were cultured as before in an environmental chamber and watered every day with one fourth strength Herridge's solution. After 14 days culture the roots were screened for nodule number.

*Analysis of nodulation: soil screening.* Regenerant shoots were placed in 50% Vermiculite™ 50% Faffard™ soil-less compost in six inch pots. These plants and 5 day old seedlings of the same mutant genotype were inoculated with 10 ml of $10^6$/ml strain USDA110 on transfer. The nodule phenotype was screened six weeks after transfer to soil.

## 3. Results

*Embryogenic regeneration system*

*Initiation of embryogenic callus.* The first attempt to initiate embryogenic tissue from cv. Peking, Bragg and nodulation mutants nts382, nts1007, nod49 and nod139 using Difco Bacto Agar (from Difco Laboratories, Detroit) and 2,4-D (from Sigma Chemicals, St. Louis) failed to produce embryogenic tissue. After personal communication with Dr. John Finer, the following modifications to the culture conditions were made. Only the pods set from the first flowers on each plant were used. The immature cotyledons were initially plated on Gelrite™ instead of Difco Bacto Agar.

Purified agar from Sigma Chemicals was used for all subsequent culture stages on solid media. The 2,4-D was purified by dissolving the hormone in 100% ethanol, followed by precipitation with one volume of water. This was followed by filtration and drying of the salt in the fume hood. The pH of the initiation medium was changed from pH 5.7 to pH 7 according to Komatsuda and Ohyama (1988). After these modifications it was possible to initiate routinely embryogenic tissue from the cultures (Fig. 1A). This suggests that embryogenesis was possible

only under stringent conditions with the purest chemicals. No experiments were performed to clarify the nature of the impurities, or whether impurities in the agar or the 2,4-D inhibited embryogenesis. Size of the immature seed plated proved significant. Immature seeds smaller than 2 mm in length died, and any seeds larger than 6 mm in length failed to produce rapidly growing callus or initiate embryogenic tissue. The optimum size for plating appeared to be 4 mm. There was significant variation in time and the number of embryogenic events occurring in the different soybean genotypes taken to initiate embryogenic tissue (Table 1).

Bragg and Fayette generally produced embryogenic callus after two months of culture and produced approximately the same number of embryo induction events. All nodulation mutants, despite being near-isogenic and from seeds derived from nitrate-grown mother plants, showed less induction events and required more time in culture to do so. Nodulation mutant nod139 did not produce any embryo induction events. Because of the low number of total initiation events, it was not clear if there was any biological significance to these observations. This observation may be due to the reduced fitness of these mutants in culture. Often the explant would appear dead or darkened before embryogenic tissue was induced (Fig. 1A).

Table 1. Time in culture before initiation of embryogenic tissue from cv. Peking, Bragg and nodulation mutants nts382, nts1007, nod49 and nod139. The embryogenic tissue was produced by culturing immature cotyledons (10 plates, 10 cotyledons per plate) on a medium containing very high levels of 2,4-D (40 mg/l). It appears that most embryogenic tissue was produced in the two to five month period after the initiation of culture. At best, 9% of cotyledons produced embryogenic tissue.

| Genotype | Number of cotyledons which developed embryogenic tissue<br>Months in culture | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Total |
| Peking | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| Bragg | 0 | 1 | 3 | 2 | 2 | 1 | 0 | 0 | 9 |
| nts1007 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| nts382 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 3 |
| nod49 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| nod139 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Fayette | 0 | 3 | 3 | 1 | 0 | 0 | 0 | 0 | 7 |
| Total events/month | 0 | 4 | 6 | 8 | 3 | 1 | 0 | 0 | 22 |

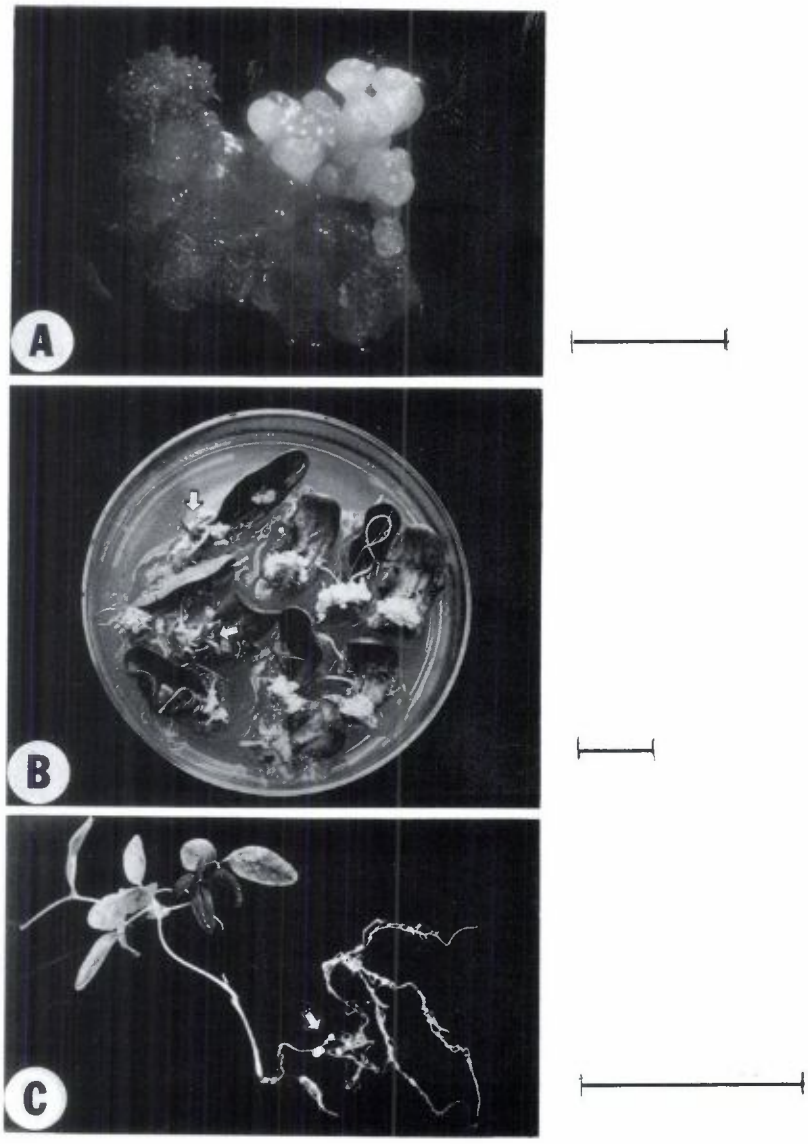


Figure 1. **A.** Somatic embryos forming on an immature cotyledon of supernodulation mutants nts1007 on embryo induction medium. Bar = 20 mm.
**B.** Callus, roots and shoots induced with 5 μM BAP on cotyledonary petioles of cultivar Peking. Arrows show regenerating shoots in the peticolar region. Bar = 10 mm.
**C.** A regenerant of nts382 produced by the cotyledonary petiole system using 150 μM BAP. This plant demonstrated a supernodulating phenotype. Arrow shows nodules on roots of regenerant. Bar = 30 mm.

*Growth in suspension culture.* The embryogenic suspension also varied in their viability in culture. Bragg and nts1007 produced healthy early stage embryogenic suspension cultures similar to those reported for the cultivar Fayette. Peking and nod49 produced embryogenic callus but it did not thrive in the liquid suspension medium. Supernodulating mutant nts382 embryogenic suspension cultures seemed to consist of larger green 'later staged' embryogenic clumps. This stage of embryonic tissue was not ideal for liquid culture. Increasing the 2,4-D level did not improve the cultures. Maintaining the cultures at a very low inoculum was of advantage.

Embryo germination capacity varied between the mutant types. Bragg and nts1007 germinated relatively quickly, whereas nts382 embryos did not develop and germinate normally. When placed on the embryo development medium, the nodulation mutant nts1007 produced a mixture of funnel-shaped and normal embryos. A proportion of these funnel embryos did not possess normal apical meristems. The germination rate from these funnel-shaped embryos could be increased by culturing with ABA. We used 10 μM ABA in the embryo development medium for 2 weeks prior to desiccation (data not shown). The ABA helped 'normalize' the embryos increasing the total number of embryos with two cotyledons and an active apical meristem.

*Regeneration of plants from embryogenic suspension cultures.* Plants from embryogenic suspension cultures of only mutant nts1007 have been regenerated. It was extremely important to wait until the embryos reached physiological arrest before transferring them to the desiccation stage. Physiological arrest was indicated by the embryos turning from green to yellow. Early in the procedure many green embryos were wasted in the desiccation stage. Germination of the somatic embryos took 8 to 15 days. Approximately 2–5 days were required for root formation and 7–10 days for the shoot formation. After transfer to soil and a period of slow growth due to tissue culture carry-over, the nts1007 plants developed into normal looking healthy fertile plants with a supernodulating phenotype. The nts1007 supernodulation phenotype did not show somaclonal variation (Larkin et al., 1984) after embryogenic regeneration.

### *Cotyledonary node organogenic regeneration*

Initial experiments attempted to repeat the results of Wright et al. (1986a; b) and Hinchee et al. (1988) with organogenesis of shoots from cotyledonary petioles of the nodulation mutants using 5 μM BAP. The Bragg derived soybean mutants did not produce any adventitious shoots at this hormone level. Cultivar Peking, however, displayed multiple shoot organogenesis with 5 μM BAP (30 out of 50 explants with up to 10 plantlets per successful explant), confirming the previously published results.

Shoot organogenesis from the cotyledonary nodes of the Bragg derived nodulation mutants

If no BAP was included in the culture medium of Bragg and the derived nodulation mutants the axillary shoots (if present) and roots grew out from the cotyledonary petiole. With 5 μM BAP in the culture medium rooting was reduced but only the axillary shoots (if present) grew out of the petiole. No shoot organogenesis occurred with Bragg and the nodulation mutants at 5 μM BAP. As the explants produced root growth at 5μM BAP this indicated that the cytokinin level was too low to initiate shoot organogenesis. Further experiments were initiated with elevated BAP levels to stimulate shoot organogenesis. The experiments were repeated with two different ranges of BAP: 0 to 300 μM BAP and 0 to 800 μM BAP (Table 2). In the latter experiment, Bragg seed (from the USDA) and Essex seed were compared in the cotyledonary node system to our Bragg seed (derived from Australian seed stock) and the nodulation mutants. The comparison between the different seed sources for Bragg allowed us to evaluate if any differences in our seed stock.

Peking produced multiple shoots from the cotyledonary node over a wide range of BAP concentrations (5 to 800μM) (Fig. 1B). In contrast, the level required for shoot initiation in Bragg, nts382, nod49 and nod139 was at least 25 μM BAP. However, most of the soybean genotypes fell within an optimum range of BAP concentrations.

Table 2. Mean number of adventitious shoots initiated from different soybean genotypes. Cultivars Peking, Essex, Bragg and the nodulation mutants were cultured on a $B_5$ medium containing a range of BAP concentrations (± standard error). Ten cotyledons per cultivar were plated with two cotyledons per Petri dish. In-house Bragg seed (1) was compared to Bragg seed obtained from USDA seed stocks (2). Optima are indicated in bold type.

| | BAP concentration (μM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Genotype | 0 | 5 | 25 | 50 | 100 | 200 | 400 | 800 |
| Peking | 0 | 6.7±1.1 | 8.8±1.4 | 17.6±1.2 | 16.8±2.7 | **33.5±2.8** | 9.7±2.5 | 8.4±1.6 |
| Bragg (1) | 0 | 0 | 5.5±0.8 | 24.8±2.8 | **33.0±4.7** | 6.2±2.3 | 3.5±1.8 | 0 |
| Bragg (2) | 0 | 0 | 4.1±0.5 | 12.3±0.7 | **24.4±3.1** | 7.7±2.1 | 1.4±0.8 | 1.0±1.0 |
| nod139 | 0 | 0 | 5.0±1.0 | **24.0±1.2** | 10.7±2.7 | 2.9±1.0 | 1.3±0.7 | 1.7±1.2 |
| nod49 | 0 | 0 | 0 | 6.3±0.8 | **13.4±1.9** | 10.3±1.3 | 6.2±1.8 | 0 |
| nts382 | 0 | 0 | 10.0±2.4 | 13.5±3.0 | **22.0±2.5** | 7.9±2.0 | 3.3±1.5 | 0 |
| Essex | 0 | 0 | 4.0±1.2 | **15.1±0.9** | 6.5±1.6 | 7.4±2.0 | 4.3±1.2 | 1.1±0.8 |

This optimum range for regeneration of shoots was 50–100 μM BAP, except nod139 which exhibited optimum regeneration at 50 μM BAP. The frequency of shoot production in these genotypes was reduced at 400 μM BAP. Regeneration of plantlets from shoots that were induced on such high levels of BAP was not difficult and resultant plants were fertile and appeared to be of normal phenotype.

*Nodulation phenotype of cotyledonary node derived shoots.* Plants regenerated from tissue-culture showed stable nodulation phenotypes. These tissue-culture derived plants, when compared to seed-derived plants, exhibited slower root and shoot growth. This was evident in growth pouch analysis of nodulation phenotype (Table 3), where the nodule number of the tissue-cultured plants was approximately half that of the seedlings. *Bradyrhizobium* infects only actively growing roots. Thus, slow root growth limited the frequency of nodules formation. It generally took one month before the regenerated plants began growing at a normal rate in the greenhouse.

Nodule number was subsequently screened on larger plants grown in soil for six weeks after inoculation and their transfer from culture. Control plants were screened six weeks after germination and inoculation. There was no significant difference in the mean number of nodules produced from tissue culture derived and seedling derived plants (Table 4).

By screening the nodulation phenotype 6 weeks after inoculation the plant growth habits and nodule numbers of the tissue culture and seed-derived plants

Table 3. Mean nodule number from seedling and tissue culture derived plants grown in plastic pouches. The nodule numbers (± standard error) were compared 14 days after each root was inoculated with 100 μl of *Bradyrhizobium japonicum* USDA110 ($1 \times 10^6$ $ml^{-1}$). Plants were grown in controlled environment cabinets and watered with 1/4 strength Herridge's solution. One tissue-cultured and one seed derived plant were placed in each pouch. Ten pouches of each cultivar were maintained.

| Cultivar | Nodule number per plant | |
|---|---|---|
| | On seed derived plants | On tissue culture derived plants |
| Peking | 18.3±3.2 | 9.7±1.5 |
| Bragg | 18.0±2.6 | 8.1±1.3 |
| nod139 | 0 | 0 |
| nod49 | 0 | 0 |
| nts382 | 60.5±7.9 | 39.2±7.75 |

Table 4. Mean nodule number of seedling and tissue culture derived plants grown in soil for six weeks. The nodule numbers (± standard error) of 10 seed and 10 tissue culture derived plants were compared six weeks after inoculation with 10 ml of *Bradyrhizobium japonicum* USDA110 ($1 \times 10^6$ ml$^{-1}$).

| Cultivar | Nodule number per plant | |
|---|---|---|
| | On seed derived plants | On tissue culture derived plants |
| Peking | 28.6±3.2 | 29.4±4.2 |
| Bragg | 28.0±4.1 | 32.2±4.7 |
| nod139 | 0 | 0 |
| nod49 | 0 | 0 |
| nts382 | 313.6±40.3 | 316.0±32.5 |

were almost directly comparable. The pouch-grown plants were slow growing and nodulated poorly. In contrast plants grown in the soil nodulated at a normal level (Fig. 1C) and grew into vigorous fertile plants. This demonstrated that the nodulation phenotype of these tissue culture regenerant plants was not adversely affected by passage through a tissue culture system.

## 4. Discussion

Conditions were found for the regeneration of soybean cultivar Bragg and its near-isogenic nodulation mutants. Two tissue culture systems were used to induce 1) organogenic and 2) embryogenic regeneration in these soybean genotypes. The cotyledonary petiole system was used for organogenic shoot induction on the petioles of seedling cotyledons and these shoots were later regenerated into plants. The embryogenic system involved the induction of somatic embryos on immature cotyledons; the initiation of embryogenic suspension cultures from the embryos; and the regeneration of plants from proliferating embryos. The embryogenic system had only limited success compared to the cotyledonary petiole system when regeneration of plants was attempted. Plants regenerated from the tissue culture systems were tested for their ability to nodulate with *Bradyrhizobium japonicum*, considering their parental nodulation genotype.

The cotyledonary petiole system proved efficient for induction of organogenic shooting ands subsequent regeneration for Bragg and the supernodulating and non-nodulating mutants. In this system high concentrations (50–100 μM) of

benzylamino purine (BAP) were necessary for optimal shoot organogenesis. This was compared to a BAP concentration of 5 μM for the control cultivar Peking. These results suggested that there can be marked differences in cultivar response to exogenously applied cytokinin in this culture system. Our results also reflect a higher hormone concentration requirement for shoot organogenesis on $B_5$ media as compared to MS media. This effect was seen by both Cheng et al. (1980) and Saka et al. (1980). This system was more rapid than the embryogenic system for the production of regenerated plants (3–4 months).

Embryogenic suspension cultures of Bragg, supernodulating nts382 and nts1007, and non-nodulating nod49 and nod139 were initiated. Embryos from these suspension cultures were matured, desiccated, and germinated in vitro in attempts to regenerate plants. Initiation of embryogenic cultures from these cultivars took significantly longer when compared to cv. Fayette, the model cultivar for suspension work. Improvements in initiation of embryogenic tissue were observed when the medium was modified with purified agar, purified 2,4-D and pH 7. The size of immature seed harvested also seemed to affect success in culture. Only supernodulating genotype nts1007 was regenerated using this system. These results suggest variable cultivar response with the use of this culture system for the regeneration of soybean plants.

The nts1007 regenerants from the embryogenic system and the Bragg and nodulation mutant plants regenerated from the organogenic system were fertile and showed the same nodulation phenotypes of the parental material. This was an important observation as nodulation is known to be affected by hormones (Caetano-Anollés and Gresshoff, 1991; Gresshoff, 1993). It was thus feasible that the tissue culture treatments and the culture of root tissues for long periods on nitrate-containing medium would alter the nodulation phenotype. Regenerated Fayette plants have been known to become infertile after passage through the embryogenic system, presumably from genetic changes in culture (Dr. J. Finer, personal communication). Our results here suggest that the nodulation phenotype of the regenerants did not change after passage through an organogenic or an embryogenic (only nts1007) tissue culture regeneration system.

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