EFFECT OF BENZYLAMINOPURINE AND CULTIVAR ON PLANT REGENERATION FROM SOYBEAN COTYLEDONARY NODES IN CULTURE

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CHAPTER I

INTRODUCTION

Today it is generally accepted that the term 'plant tissue culture' broadly refers to the cultivation *in vitro* of all plant parts, whether a single cell, a tissue, or an organ, under aseptic conditions. Plant tissue culture, as a technique, is becoming increasingly important for the investigation of plant growth and development as well as for practical application to agriculture. One goal of tissue culture is the regeneration of plants *in vitro*. Tissue culture-derived plants could be used in breeding programs along with those produced by more conventional methods.

Regeneration of plants from tissue culture has been accomplished with a sufficient number of species to assume that, theoretically, it can be done with all plants. However, reports on tissue culture of legumes are scarce. The work described in this report extends previously reported work on soybeans (*Glycine max* L.).

CHAPTER II

LITERATURE REVIEW

In vitro legume cultures were first established in the 1950's by Nickell (9). Since then callus and cell suspension cultures of various legumes have been reported (1,5,8,10,11,13,14). Regeneration of legume plants has been reported infrequently. Saunders and Bingham (13) first reported the culture and successful redifferentiation of plantlets from alfalfa (*Medicago sativa* L.). Scowcroft and Anderson (14) induced plant regeneration from the tropical legume *Stylosanthes hamata*. Phillips and Collins (11) established callus cultures and initiated plant regeneration from red clover (*Trifolium pratense* L.).

In the case of soybean, callus and cell suspension cultures have been established and under investigation for many years (1,2,5,6,11). Soybean is conspicuously absent from the list of species that have undergone *in vitro* regeneration, however.

Soybean is similar to other legume species in that the ability to regenerate plants seems to be lost in callus cultures. Street (16) suggested that for propagation purposes the period of unorganized growth (callus) should be minimized or, even better, it should be eliminated entirely. Hicks (4) pointed out that callus formation is an undesirable complication in that first, the chromosome constitution of callus cells is unstable in many plants. Secondly, the ratio of potentially organogenic (meristemoid) cells to the total callus mass is very small.

Furthermore, there is a lack of synchrony between individual meristemoids in a given callus.

Beversdorf and Bingham (1) extensively investigated differentiation from soybean callus culture. They found that among 56 soybean cultivars studied, only one developed embryo-like structures in liquid culture. None of these developed into plantlets. In another study, Oswald, Smith and Phillips (10) reported bud formation from soybean callus grown on medium containing a high level of kinetin, but the rate of bud formation was very low.

Since soybean has failed to regenerate plantlets from callus, another approach has been used to bypass the callus stage and use tissue explants consisting of totipotent cells. Kimball and Bingham (7) reported adventitious bud development in the absence of callus formation from soybean hypocotyl sections in culture. Cheng and Saka (3) reported that soybean cotyledonary node segments, preconditioned with a high level of BAP (benzylaminopurine) and then transferred to media containing low BAP, showed stimulated shoot growth. Saka and Cheng (12), by diminishing callus growth, were able to enhance multiple shoot formation from cultured soybean stem nodes. In their study they also found that the growth of callus seemed to interfere with the process of shoot-bud formation. In order to reduce callus growth they first placed stem node segments on a basal medium devoid of any growth regulators and then transferred them to a medium containing 0.025μM IBA (indolebutyric acid) and 5μM BAP. A reduction in callus production and enhancement of shoot-bud formation were noted.

Cheng and Saka (3) reported continuous shoot-bud formation from soybean cotyledonary nodes on a medium supplemented with a high level of

BAP. When the nodes were transferred from a high BAP to a low BAP medium, shoots started to develop.

A number of media have been used in soybean culture, but modified Gamborg's medium (5) has been used extensively and proved very satisfactory (1,3,6,7,10,12).

Skoog and Miller (15) first reported that minimal changes in the ratio of auxin to cytokinin can determine whether organogenesis occurs. However, it has not always been found that the application of an appropriate balance of hormones results in organogenesis and whole plant regeneration. A number of other factors, including the physiological state of the explant, the genotype of the donor plant, environmental effects, sequential effects of development, and optimal organic and inorganic nutrients need to be taken into account.

The objectives of the present study were to extend previous work by evaluating the responsiveness of soybean cotyledonary node segments to the in vitro stimulation of various levels of BAP and to evaluate the effect of different cultivars on growth and plant regeneration from cotyledonary node cultures.

CHAPTER III

MATERIALS AND METHODS

Plant Materials

Six cultivars of soybean (Glycine max L.) (RA501, RA604, Crawford, Cumberland, Mitchell and Miles) were included in this study. Seeds were surface-sterilized in 70% ethanol for three minutes and then placed in 10% clorox (sodium hypochlorite) for ten minutes and rinsed three times with sterile distilled water. Sterilized seeds were placed on a modified B-5 medium (5) containing 0.025μM IBA and various concentrations of BAP (i.e. 1, 5, 25 or 50μM) to germinate. After approximately four weeks, the cotyledonary node segments were excised from cultured seedlings by removing cotyledons adjacent to the stem axis, and cutting the stem and hypocotyl about 3mm above and below the node region. These node segments were subsequently cultured to study shoot-bud formation and plant regeneration.

Culture Methods

The composition of the defined basal medium used for culturing soybean seeds and cotyledonary nodes is listed in Table I. IBA at a concentration of 0.025μM and BAP at concentrations of 1, 5, 25, or 50 μM were added to the basal medium. All media were adjusted to pH 5.5 with HCl or NaOH, and solidified with 0.6% tissue culture agar. Ten ml portions of the media were placed in glass tubes and capped with

TABLE I

BASIC COMPOSITION OF MB-5 MEDIUM

Components	Concentration (mg/liter)
NaH_2PO_3	150
KNO_3	2500
$(NH_4)_2SO_4$	134
$CaCl_2$-$2H_2O$	150
$MgSO_4$-$7H_2O$	250
$FeSO_4$-$7H_2O$	25
$CoCl_2$-$6H_2O$	0.025
$ZnSO_4$-$7H_2O$	2
H_3BO_3	3
KI	0.75
$MnSO_4$-$4H_2O$	10
Na_2MoO_4-$2H_2O$	0.25
$CuSO_4$-$5H_2O$	0.025
Thiamine-HCl	10
Pyridoxine-HCl	1
Nicotinic Acid	1
Inositol	100
Sucrose	20000
Agar	6000

plastic caps. The media were autoclaved at 120°C for fifteen minutes.

Initially, seeds were cultured on the media with 1, 5, 25 or 50 μM BAP to evaluate the effects of BAP on germination. Subsequently, excised cotyledonary node segments were either subcultured on the same medium as the original ones, or transferred to a medium in which IBA was still maintained at 0.025 μM but BAP was reduced to 1 μM, so as to compare the effect of reduced BAP concentration on bud growth. After shoots developed, cotyledonary nodes were transferred to a rooting medium which was devoid of plant growth regulators to induce root formation. Cultures were grown in a constant temperature room (28°C) with eight hours of dark and sixteen hours of light at an intensity of 2000 lux. Each treatment consisted of at least ten cultures and each experiment was repeated four times. Each experimental group was examined weekly with major characteristics being recorded four weeks after transfer.

CHAPTER IV

RESULTS AND DISCUSSION

Results

Soybean seeds cultured on a MB-5 medium responded differently to the different BAP treatments. When seedlings were grown on a medium containing 1 μM BAP two auxillary shoots developed from the cotyledonary region. Roots also developed vigorously with this treatment. In contrast, both shoot development and root growth were suppressed in treatments with BAP at high levels. BAP at concentrations of 25 and 50 μM resulted in stunted shoot-bud formation in the node region without further elongation. Root growth was inhibited to such a degree that no secondary root growth was found in the treatments with BAP concentrations of 25 or 50 μM. Enlarged node regions were observed in high BAP treatments. Six cultivars showed the same response to each of the treatments tested. The growth behavior of seedlings is shown in Figures 1 and 2. Apparently, the germination rate was not affected by BAP treatments since nearly 90% germination rate was obtained for six cultivars in each of the BAP treatments.

As mentioned earlier, the soybean cotyledonary node segments were obtained from cultured seedlings which had been pretreated with different concentrations of BAP. The cotyledonary node segments excised from cultured seedlings were subcultured either to new medium with the same BAP concentration or to a medium containing 1 μM BAP. The response to

Figure 1. Soybean Seeds Cultured on MB-5 Medium Containing 1 μ M BAP Showed Shoot and Root Growth.

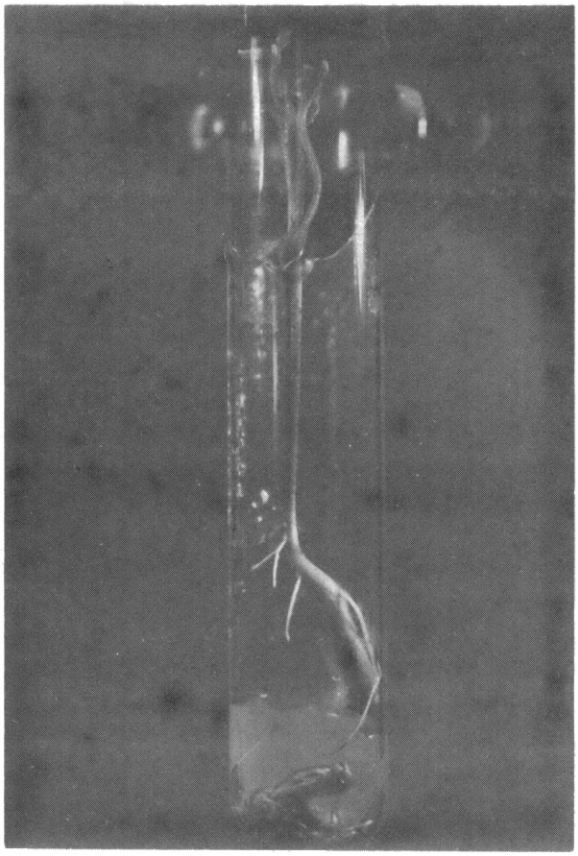
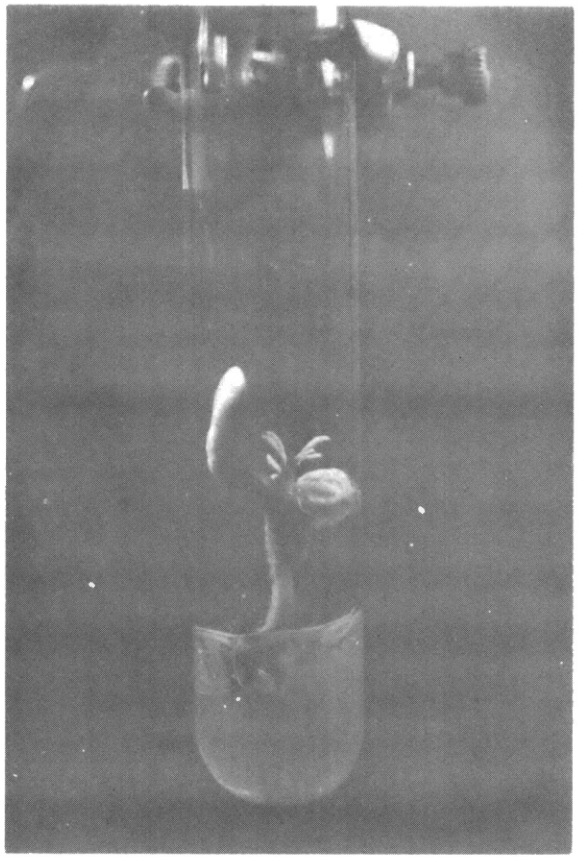


Figure 2. Soybean Seeds Cultured on MB-5 Medium Containing 25 μM BAP Showed Stunted Shoot-bud Formation.



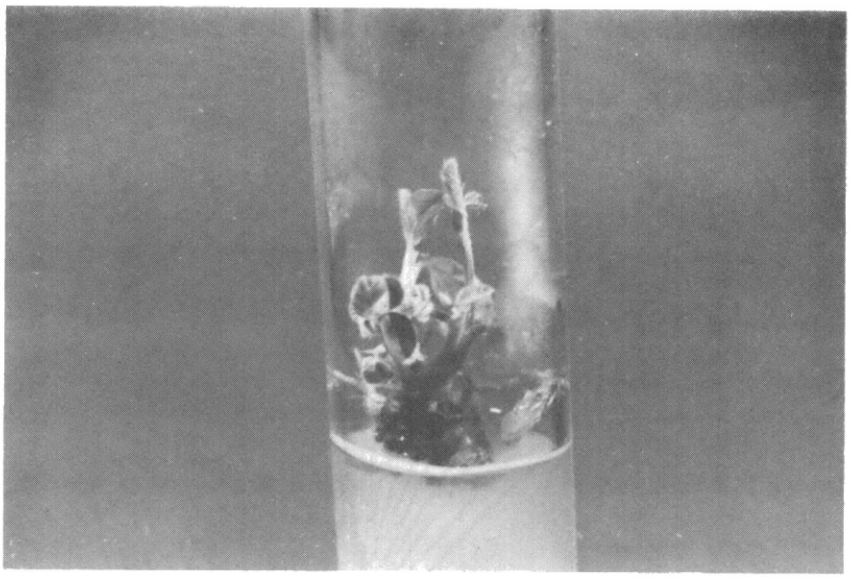
reduced BAP was very profound. Stimulation of shoot growth was observed in subcultures transferred from high BAP treatments (i.e., 25, 50 μM) to 1 μM BAP (Figure 3). Among the six cultivars studied, Crawford, Cumberland and Miles appeared to be more sensitive to the reduced BAP treatment. Vigorous shoot growth was often observed in these cultivars. It was interesting to find that Miles usually formed callus at the base of the cotyledonary node where it was in contact with the medium. Callus formation, however, did not suppress shoot growth. Cultivars RA501, RA604 and Mitchell seemed to be less responsive to the reduced BAP treatment, although shoot development was observed. The results are shown in Table II.

On the other hand, subcultures of cotyledonary node segments from high BAP concentrations of 25 and 50 μM to fresh new medium containing the same BAP levels resulted in a very different response. Numerous small shoot-buds formed on the cotyledonary node region without further growth (Figure 4). Continued shoot-bud formation was observed throughout the culture period. All six cultivars showed the same tendency to form multiple small shoot-buds. Shoot growth was not found in all cultures among any of the six cultivars. Callus formation in conjunction with numerous small shoot-bud formation was observed in cultivar Miles (Figures 5 and 6).

When node segments forming multiple shoot-buds were subsequently transferred to a low BAP concentration of 1 μM, stimulation of shoot growth occurred sporadically among all six cultivars. Unlike cotyledonary nodes directly transferred from high BAP pretreatment to low BAP medium, cultivars Crawford and Cumberland did not show vigorous shoot growth. They behaved like the other cultivars in that the frequency of cultures showing shoot growth was very low. The

Figure 3. Subcultured Cotyledonary Nodes With Shoot Growth. Cotyledonary nodes which were excised from seedlings cultured in high BAP were subcultured in medium with a reduced concentration of BAP.

Figure 4. Subcultured Cotyledonary Nodes With Numerous Shoot-bud Formation. Cotyledonary nodes excised from seedlings cultured in high BAP were transferred to the same medium.



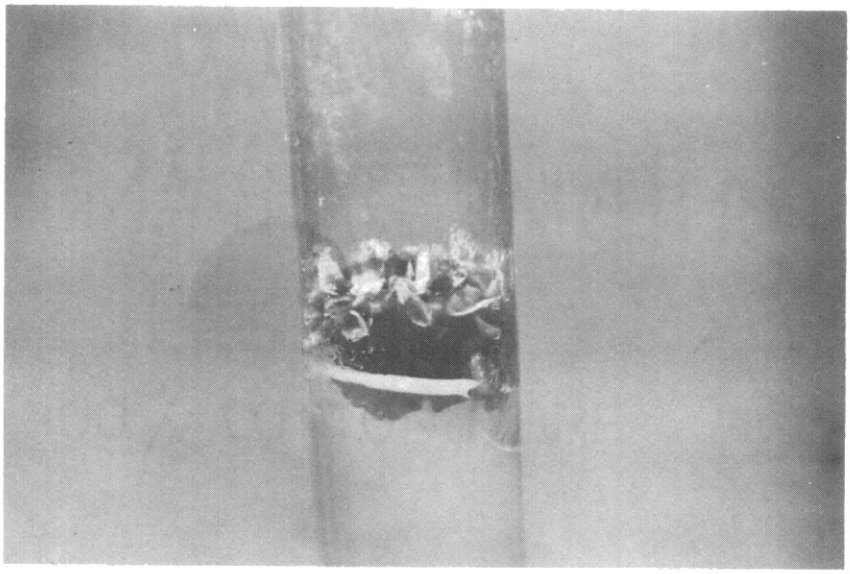
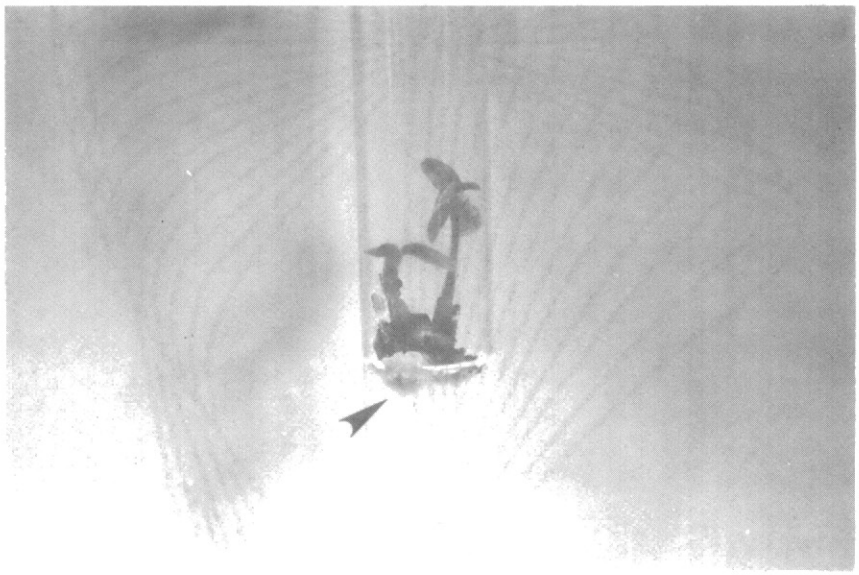


Figure 5. Shoot Growth Accompanied With Callus Formation from Cotyledonary Nodes of Cultivar Miles. Arrow indicates callus formation. Shoot growth was not suppressed by callus formation.

Figure 6. Numerous Shoot-bud Formed in Conjunction With Callus Formation from Cotyledonary Nodes of Cultivar Miles. Arrow indicates callus formation.



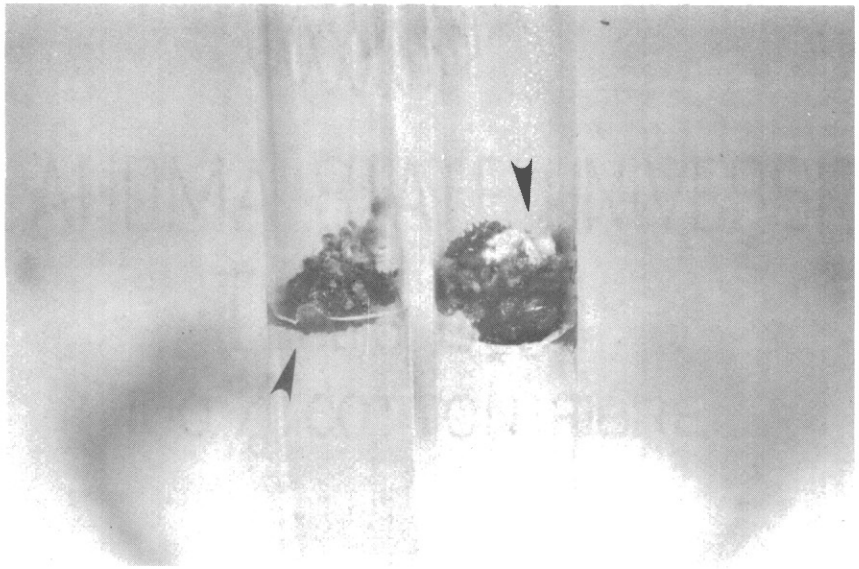


TABLE II

EFFECT OF REDUCING BAP ON SHOOT FORMATION FROM COTYLEDONARY NODES OF DIFFERENT CULTIVARS. COTYLEDONARY NODES EXCISED FROM SEEDLINGS CULTURED IN MEDIUM CONTAINING 25 μM BAP WERE SUBCULTURED IN MEDIUM WITH 1 μM BAP.

Cultivar	Percent of Cultures Developing Shoots	Mean Number of Shoots Per Culture	Callus Response
RA 501	32% (10/31)[a]	4 (2)[b]	-[c]
RA 604	22% (9/41)	6 (1)	-
Crawford	69% (25/36)	5 (2)	-
Cumberland	59% (23/39)	5 (2)	-
Miles	55% (18/33)	4 (2)	+
Mitchell	23% (10/43)	3 (1)	-

[a]The fractions of cultures that developed shoots.

[b]Mean number of shoots that exceeded 3cm in length after four weeks in culture.

[c] -: no callus, +: callus formation.

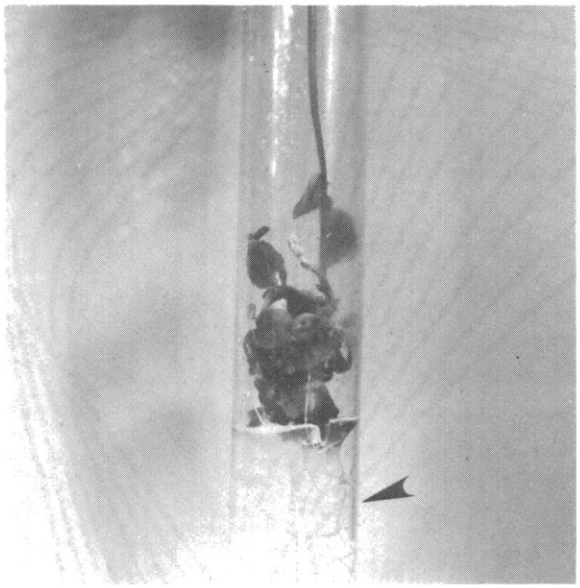
separation of multiple shoot-bud clusters prior to subculture seemed to be effective in stimulating shoot growth since some separated shoot-buds showed shoot growth. In another study, cotyledonary node segments excised from seedlings pretreated with 1 μM BAP were subcultured on the same medium. Most of the cultures either became brown or did not show any growth response. Exceptions of limited shoot growth were observed in cultivars Cumberland and RA604.

Shoots derived from cotyledonary node segments were subsequently transferred to a rooting medium to which no plant growth regulator was added. Root growth was observed among six cultivars (Figure 7). The time course of root formation varied among the six cultivars tested. Crawford tended to show root formation earlier than the other cultivars, although eventually root growth occurred in all six cultivars. Root growth was also observed among the multiple shoot-bud clusters; however shoot growth seldom occurred among them.

Discussion

In this study, the observations concerning the nature of shoot formation in soybean cotyledonary node culture were generally in agreement with those of Cheng and Saka (3). The frequency of shoot formation was found to vary among the six cultivars. Although all six cultivars formed shoots when subcultured from high BAP pretreatment to reduced BAP medium, the percentage of cultures forming shoots varied from 69% for Crawford to 22% for RA604. The qualitative response of cultivars to varied BAP treatment indicated that no substantial difference existed among the cultivars. The major difference was in the percentage of cultures that developed shoots indicating that the

Figure 7. Root Formation from Cotyledonary Node Derived Shoots. Arrow indicates root formation.



capacity to regenerate shoots differs among the cultivars. Miles was the only cultivar which consistently showed callus formation, however callus growth did not suppress shoot development to any degree. This may be due to the low concentration of IBA present. Low IBA, as maintained in the media used, would not favor callus growth. Thus it is not surprising that callus formation did not interfere with shoot growth.

Two-step procedures to obtain plant regeneration or organ formation in tissue cultures are not uncommon (12,13,17). In this study, soybean cotyledonary node segments were derived from seedlings sown on media containing different levels of BAP and subsequently subcultured in a medium with lower BAP concentration. In the first step of this process, the cotyledonary node segments had already been activated and in the second step, the stimulation of shoot-bud growth occurred. Pretreatment with high BAP concentrations favors shoot-bud formation. If nodes are serially transferred to the same high BAP medium, additional shoot-buds proliferate. These do not continue to develop if placed on low BAP medium, however. These numerous shoot-bud forming node segments seemed to have lost responsiveness to the lowered BAP treatment. Crawford, for example, a highly responsive cultivar, did not show the same response if it was subcultured to the high BAP treatment again and then transferred to low BAP medium. Apparently, a high BAP treatment is effective in stimulation of bud formation but inhibits shoot development. Reducing the BAP concentration seemed to release the inhibition of shoot growth. However additional stimulation of shoot-bud formation enhanced the inhibition which could no longer be overcome by reducing the BAP level only. Separation of large shoot-bud clusters may have some effect in releasing the inhibition of shoot growth. Some cultures

of Cumberland did show shoot development upon separation of shoot-bud clusters. However not all cultivars were tested. More experiments are needed to verify this. Cotyledonary node segments derived from seedlings pretreated with 1 μM BAP did not show prolific shoot-bud formation when subcultured to medium containing 1 μM BAP. On the contrary, limited shoot growth was observed, but the frequency was very low and was found in cultivars RA604 and Cumberland only. It was evident that pretreatment with low BAP concentration was not effective in the stimulation of bud formation.

CHAPTER V

SUMMARY AND CONCLUSIONS

Tissue culture methods were used to study the capacity of soybean cotyledonary node segments to regenerate whole plants. Cotyledonary node segments from seedlings of the cultivars RA501, RA604, Crawford, Cumberland, Miles and Mitchell which had been sown on MB5 media containing various concentration of BAP responded differently to the lowering of BAP. Stimulation of shoot growth was observed when cotyledonary node segments that had been pretreated with high BAP (i.e., 25 or 50 μM) were subcultured on a medium with 1 μM BAP. Cultivars Crawford, Cumberland and Miles were sensitive to this treatment. Numerous shoot-buds formed when cotyledonary node segments were transferred to the same media containing a high concentration of BAP. Stimulation of shoot growth was observed when those multiple shoot-bud clusters were subcultured on media containing low BAP, however the frequency was very low. Miles was the only cultivar showing callus formation in both cases. MB-5 basal medium without plant growth regulators was suitable for induction of root growth in cotyledonary node derived shoots.

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