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The Effect of Media Constituents on In Vitro Culturing of Cowpea (*Vigna unguiculata*) Shoot Tip and Leaf Disk Explants

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Abstract

Cowpea is an important legume food crop that is commonly grown in Arkansas and numerous other southern states. The application of biotechnological approaches for the improvement of U.S. cowpea genotypes is currently not possible due to the lack of a regeneration and transformation system. Therefore, the first priority of our research efforts is the development of a plant regeneration system that will facilitate plant transformation studies. In an effort to optimize the media requirements for tissue culturing cowpea, we evaluated the in vitro response of shoot tip and leaf disk explants to various levels of Murashige and Skoog (MS) macro and micro nutrients, vitamins, and iron. One commercial cultivar, Early Scarlet (formerly 91-135), and one advanced Arkansas breeding line, 91-245, were used as tissue sources. Shoot tips were cultured on media augmented with 5 mg/L kinetin and 0.01 mg/L naphthaleneacetic acid (NAA). Multiple shoots were produced from shoot tips, and these grew well when cultured on full strength MS. However, increasing MS levels to 1.5 times the standard concentration induced taller shoots from both genotypes. Leaf disks were cultured on MS media supplemented with 0.5 mg/L benzylaminopurine (BAP) and 1 mg/L 2,4dichlorophenoxyacetic acid (2,4-D). Callus proliferation was greatest on media containing full strength MS supplemented with 0.5 mg/L BAP and 1 mg/L 2,4-D. The effects of the media constituents were genotype dependent, with Early Scarlet generally producing larger shoots and greater amounts of calli. The results obtained from this study demonstrate that the plant genotype and growth hormones have the greatest influence on cowpea growth in vitro. Therefore, in developing a cowpea regeneration system, it will be necessary to test numerous genotypes in combination with various growth regulators. To improve regeneration frequencies the media components can be optimized for the genotypes of interest.

Introduction

Cowpea, or southernpea [*Vigna unguiculata* L. (Walp.)], is a drought-tolerant grain legume that constitutes a source of dietary protein in Africa, Brazil, India, and the USA. Commercial cowpea growing areas are found in numerous states, such as Arkansas, California, Georgia, Louisiana, Mississippi, Missouri, and Texas. Constraints to U.S. production include the lack of both drought tolerance and resistance to pests and diseases. Cowpea stunt, a viral disease caused by the synergistic interaction of cucumber mosaic virus (CMV) and blackeye cowpea mosaic virus (BlCMV) can cause devastating economic losses to local farmers (Anderson et al., 1996). Unfortunately, good sources of resistance to both of these stunt viruses have not been found within the domesticated species. Therefore, the availability of a regeneration and transformation system would provide a means for producing transgenic virus resistant cowpea.

Effective molecular genetic manipulations require a reliable method of plant regeneration. However, tissue culture studies on cowpea have shown it to be highly recalcitrant, and attempts to regenerate plants from in vitro-cultured explants have not been very successful (Latunde-Dada, 1990; Morginski and Kartha, 1984). In general, the culture medium plays an important role in the ability of the explant to regenerate shoots, with the specific medium requirements being highly dependent on the plant species (Dougall, 1981; Pierik, 1993). Therefore, we initiated this study to help us understand the media requirements for cowpea, which will aid us in our efforts to regenerate cowpea.

The long-term goal of our research is to develop a system in which biotechnological approaches can be utilized efficiently to introduce agronomically important genes into local cowpea breeding lines and commercial cultivars. Ultimately techniques such as these may help breeders produce cultivars that can overcome losses due to drought, pests and diseases. The objective of this study was to identify media that would be optimal for cowpea growth in vitro using 1) the response of shoot tip explants of two cowpea genotypes to various levels of Murashige and Skoog (MS) basal salts, iron, and vitamins and 2) the callusing response of leaf disk explants of two genotypes with two auxins and various levels of MS, iron, and vitamins.

Materials and Methods

Disinfection and Culture Establishment. (Shoot tips).--Two cowpea genotypes, the commercial cultivar Early Scarlet (formerly breeding line 91-135) and the advanced breeding line 91-245, were used in this study. Seeds were surface sterilized in 70% ethanol for 1 min and then shaken for 15 min on a gyratory shaker at 100 rpm in 1.6% w/v sodium hypochlorite (30% v/v Clorox, commercial bleach) containing 3 drops of Tween 20 (Sigma Chem. Co., St. Louis) per 100 ml Clorox solution. Seeds were then cultured individually on germination medium. Shoot apices, 5 mm long, of 7-day-old seedlings were isolated and placed on culture initiation medium in 25xl50-mm tubes. Shoot tips were placed vertically on the culture medium with approximately 1 mm of the cut end inserted into the medium.

Leaf Disks.--Trifoliate leaves from 11-day-old greenhouse-grown plants, Early Scarlet and 91-245, were removed and surface sterilized in 70% ethanol for 30 s, followed by immersion in a 20% Clorox solution for 10 min. The leaves were rinsed 4 times in sterile distilled water, cut into disks using a cork borer, and cultured with the abaxial side in contact with the medium.

Culture Medium and Conditions.--The basal culture medium, which served as a reference or standard medium throughout this study, consisted of MS (Murashige and Skoog, 1962) salts supplemented with 0.2 mg/liter thymine, 80 mg/liter casein hydrolysate, 3% sucrose, 0.8% agar [Agar-agar/Gum agar] (Sigma), and the vitamins 100 mg/liter *myo*-inositol, 1 mg/liter thiamine-HCl, 1 mg/liter nicotinic acid, 1 mg/liter pyridoxine-HCl and 2 mg/liter glycine. MS macro and micro nutrients at levels of 0.25, 0.5, 0.75, 1, and 1.5, vitamins at 0.5, 1, 1.5, and 2, and ferric-EDTA at 0.5, 1, and 1.5 times the standard concentrations were tested. The shoot tip culture medium consisted of variations of the standard medium supplemented with 5 mg/liter furfurylaminopurine (kinetin) and 0.01 mg/liter naphthaleneacetic acid (NAA). The leaf disk callus induction medium was composed of variations of the standard medium augmented with 0.5 mg/liter benzylaminopurine (BAP) combined with 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA at 1 mg/liter. The media were adjusted to pH 5.8 with 1*M*KOH, dispensed in 25xl50-mm culture tubes (15 ml per tube), and autoclaved at 121° C and $1x10^5$ Pa (1.1 kg cm^{-2}) for 15 min. Shoot tip cultures were maintained at 12-h photoperiods of cool-white fluorescent light and 23±2°C, whereas, leaf disks were maintained under continuous dark conditions and 23±2°C.

Statistical Analysis.--This study consisted of six experiments, each with 9 replications per treatment. The first three experiments utilized shoot tips as explants and were set up as a two factor factorial, the two factors consisting of genotypes (Early Scarlet and 91 -245) and one of the three media components (MS, iron, or vitamins). After three weeks, data were recorded on height of shoots (cm), number of leaves per explant, number of shoots per explant, and the size of the largest leaf (cm). The second three experiments utilized leaf disks as explants and were set up as a three-factor factorial. The factors were genotype (Early Scarlet and 91-245), hormone type (NAA or 2,4-D), and one of the three media components (MS, iron, or vitamins). After a culture period of 3 weeks, data were taken on callus mass (g).

Data were subjected to an analysis of variance for a completely randomized design. Trend analysis was done in which the variation due to rates was partitioned into linear, quadratic, and cubic sources of variation. The linear source is the average change in the response as the medium concentration increases; quadratic and cubic sources measure degrees of departure from a straightline response. Statistical significance of any of these trend components indicates that the true response means differ with all medium concentrations. If the linear source is the only significant trend component, then a straight line is the pattern of response to increasing medium levels; if the non-linear sources are also significant, then the change in response is dependent upon the medium concentration. Mean separation was done by a T-test and significance was determined at the 5% significance level.

Results and Discussion

Effect of MS Major and Minor Nutrients on Shoot Tip Growth.--Varying the concentration of the MS macro and micro nutrients in the culture medium influenced the in vitro growth of shoot tips, with the responses differing between genotypes. There were significant linear and quadratic effects on shoot height for Early Scarlet and 91-245. Increasing MS levels up to 1.5 times the standard concentration resulted in taller shoots for both cowpea lines (Fig. 1A). However, Early Scarlet produced significantly taller shoots than 91-245 at all MS levels. Miller and Murashige (1976) also reported an increase in shoot height of foliage plants when the MS nutrients were increased over that normally provided in the medium. Interestingly, Early Scarlet shoot tips that were cultured with 1.5 MS also produced roots (Fig. 2). Since maximum shoot height for both lines was achieved with the highest MS level, 1.5 times the standard concentration, a further increase in the MS level may also further stimulate shoot growth.

Statistical analysis of data indicated a linear and quadratic significance for the number of leaves per explant with both Early Scarlet and 91-245. Increasing the MS level from one quarter to the standard concentration yielded the maximum number of leaves, 10 per explant, for Early Scarlet (Fig. I B). A similar significant increase in the number of

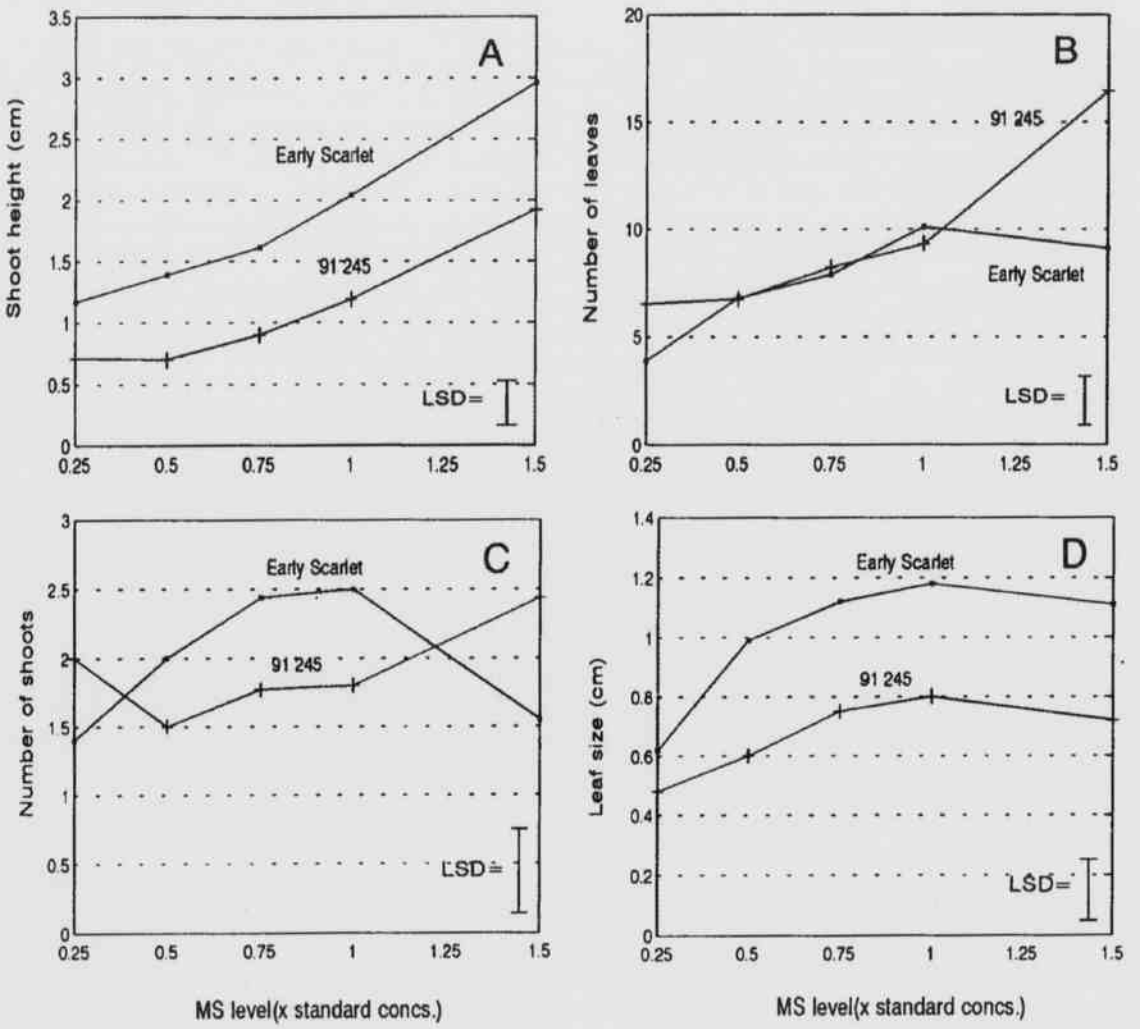

Fig. 1. Effect of MS levels on A) shoot height, B) number of leaves per explant, C) number of shoots, and D) size of the largest leaf for cowpea shoot tips derived from genotypes Early Scarlet and 91-245.

leaves produced was also observed for 91-245. However, unlike Early Scarlet, a further increase in the MS level, up to 1.5 times the standard concentration, resulted in an increase from 9 to 17 leaves per explant for 91-245.

There was a significant quadratic effect for the number of shoots per explant with Early Scarlet, whereas, both the linear and quadratic effects were significant for 91-245. Similar numbers of shoots were produced from explants cultured on media containing MS levels of 0.5, 0.75, and 1 times the standard concentration, however, with 1.5 MS the explants produced more shoots (Fig. 1C). The maximum number of shoots for Early Scarlet was obtained for shoot tips cultured on full strength MS.

The size of the largest leaf was linearly and quadratically significant for both genotypes. Maximum leaf sizes for both Early Scarlet and 91-245 were obtained with full strength MS (Fig. 1D). At this MS level, Early Scarlet produced significantly larger leaves than 91-245.

Effect of Iron on Shoot Tip Growth.--The linear response was significant for the height of shoots for both Early Scarlet and 91-245. Increasing the iron level in the medium reduced the shoot height for both Early Scarlet and 91-245 (Fig. 3). However, Early Scarlet produced taller shoots for each iron level. The number of leaves, number of

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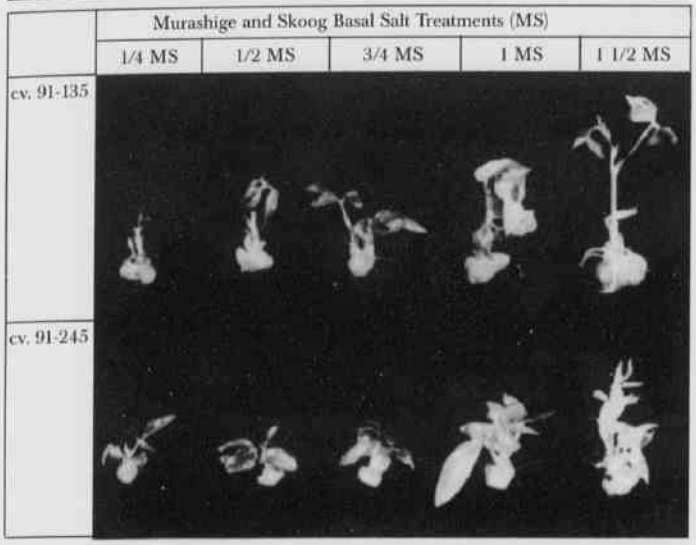

Fig. 2. Effect of MS levels on in vitro shoot growth of cowpea genotypes Early Scarlet (formerly breeding line 91-135) and 91-245.

shoots, and the size of leaves were not significantly influenced by the level of iron in the medium. However, leaf yellowing was observed with shoot tips cultured on medium containing 0.5 ferric-EDTA, indicating a deficiency of iron.

Effect of Vitamins on Shoot Tip Growth.--Varying the vitamin level in the medium produced no significant effect on shoot height or leaf size. The cowpea line Early Scarlet, however, produced taller shoots (mean = 2.76 cm) (Fig. 4) and larger leaves (mean = 1.16 cm) than 91-245 (shoot height mean = 1.42 cm; leaf size mean = 0.81 cm).

The cultivar by treatment interaction was significant for the number of leaves produced per explant. Increasing the level of vitamins resulted in an increase in the number of leaves for Early Scarlet (Fig. 5A). In 91-245 the maximum number of leaves per explant was achieved with a vitamin level of 1.5.

Increasing the level of vitamins in the medium had no significant effect on shoot multiplication for Early Scarlet. When vitamin levels were varied (Fig. 5B), data analysis revealed a significant linear and quadratic response on number of shoots produced per explant for 91-245. Increasing

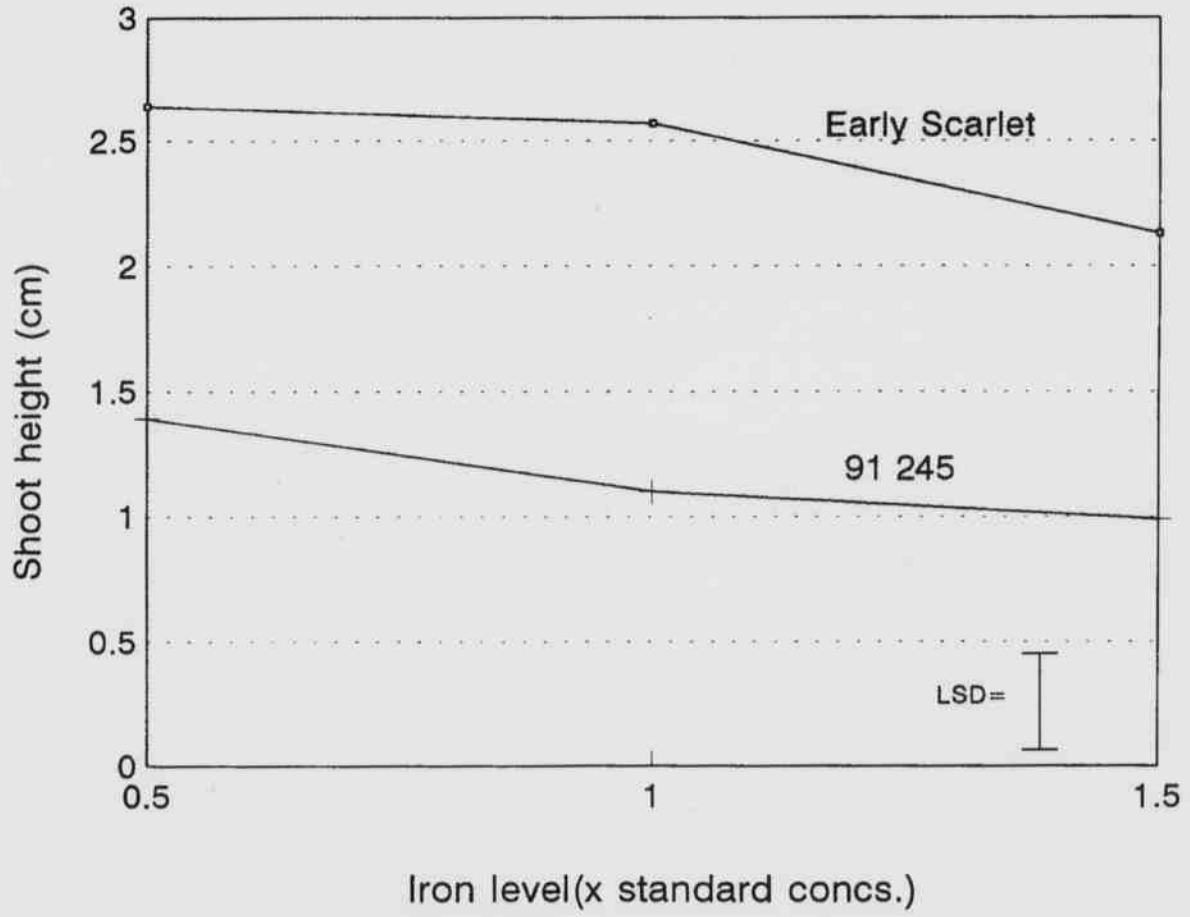

Fig. 3. Effect of ferric-EDTA on shoot height of cowpea shoot tips, Early Scarlet and 91 -245.

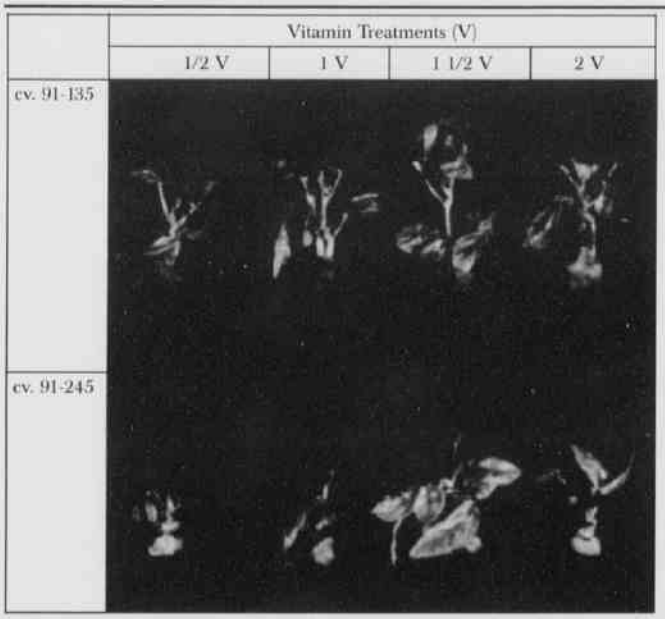

Fig. 4. Effect of vitamin levels on shoot tip culture of cowpea genotypes Early Scarlet (formerly breeding line 91-135) and 91-245.

the level of vitamins in the medium significantly increased the number of shoots produced.

Effect of MS Macro and Micro Nutrients on Callus Induction.--The amount of callus produced from leaf disk explants was influenced by the MS level, auxin type, and genotype. There was a significant linear and quadratic responses for callogenesis using both Early Scarlet with 2,4-D or NAA, and 91-245 with 2,4-D or NAA. Increasing the MS level in the medium resulted in greater callus growth to a maximum with full strength MS, regardless of auxin type or genotype (Fig. 6). Increasing the MS level from full to one and a half strength inhibited callus growth. At full strength MS, media augmented with the auxin 2,4-D stimulated greater callus growth than media containing NAA in both Early Scarlet and 91-245. On media containing all levels of MS, except 1.5 times the standard concentration, Early Scarlet produced more callus than 91-245, regardless of the type of auxin present in the media. The auxin NAA stimulated rhizogenesis; whereas, 2,4-D inhibited the production of roots in all treatments (Fig. 7). The superiority of NAA over 2,4-D in stimulating rhizogenesis was also reported for cowpea cv. Georgia-21 (Brar et al., 1997).

Effect of Vitamins on Callus Induction.--There was a significant cultivar by hormone interaction for callus induction from leaf disks. The greatest amount of callus was produced with leaf disks from Early Scarlet cultured on media

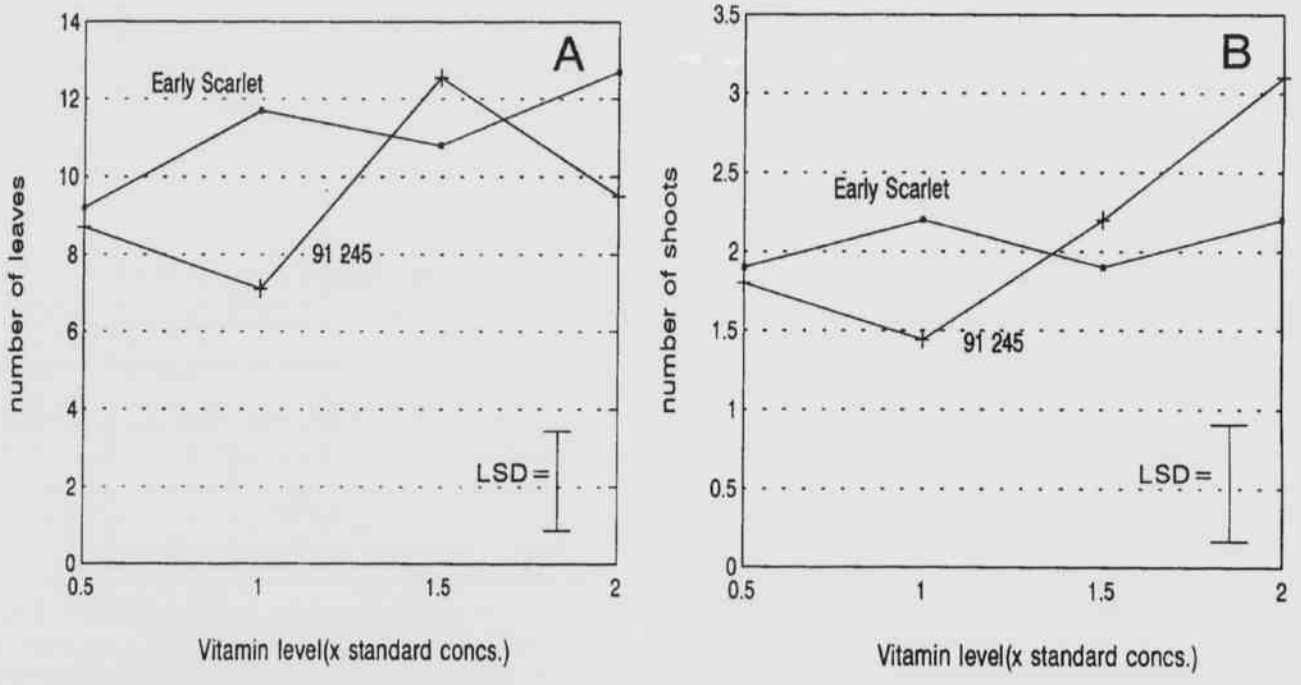

Fig. 5. Effect of vitamin levels on the A) number of leaves and B) number of shoots, produced from shoot tip culture of two cowpea genotypes, Early Scarlet and 91-245.

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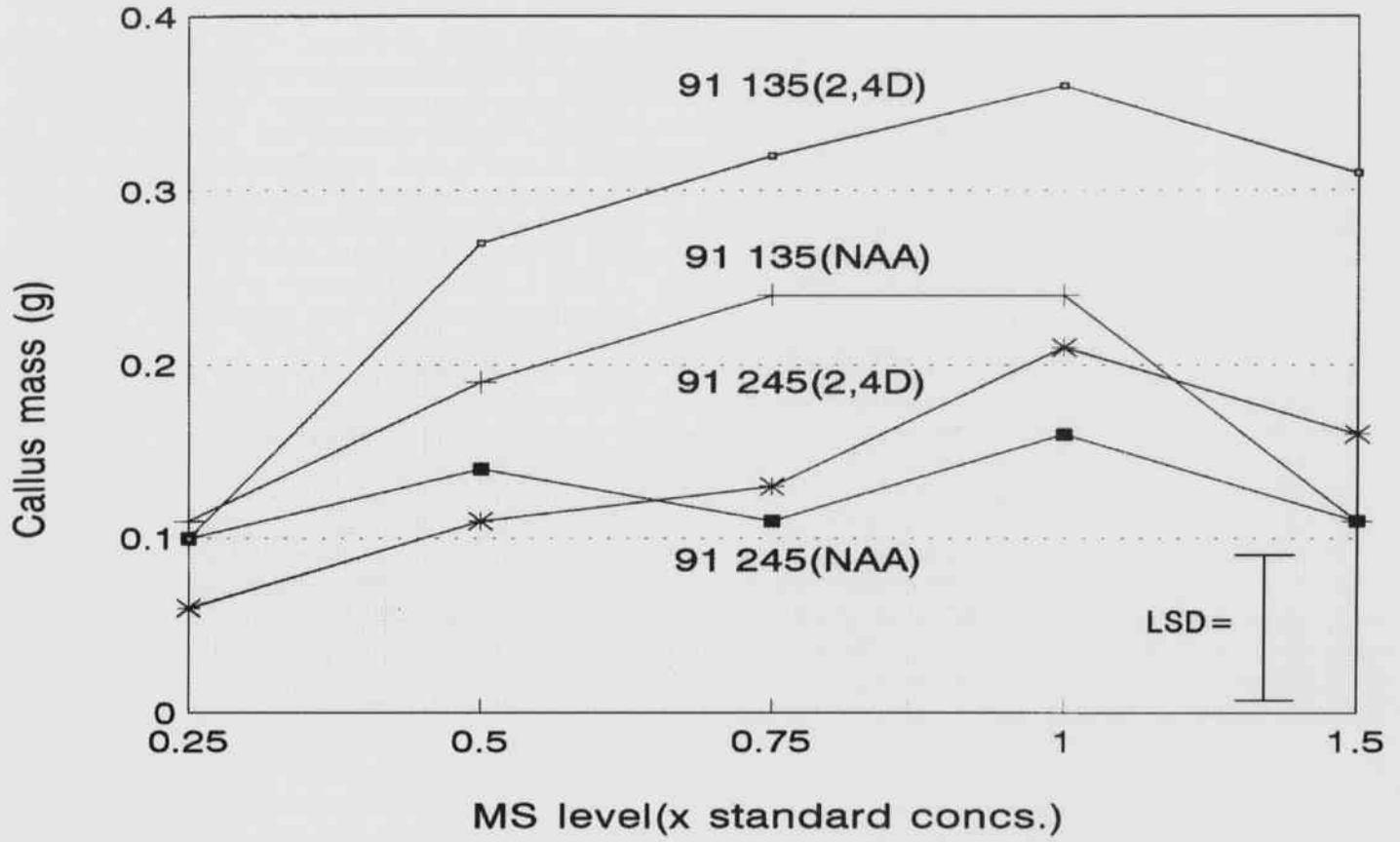

Fig. 6. Effect of MS levels, auxin type (2,4-D or NAA), and genotype (Early Scarlet or 91245) on callus induction of cowpea leaf disk explants.

Table 1. Effect of auxin type (2,4-D or NAA) and genotype (Early Scarlet or 91-245) on callus induction of cowpea leaf disk explants.

Plant Genotype	Auxin	Callus Mass (g)
Early Scarlet	2,4-D	0.30[a]
	NAA	0.20[b]
91-245	2,4-D	0.13[c]
	NAA	0.16[bc]

supplemented with 2,4-D (Table 1). In fact, Early Scarlet leaf disks produced greater amounts of callus than those of 91-245, regardless of the auxin type. The level of vitamins in the medium did not significantly affect callus growth (data not shown). Matsubara (1975) tested the effects of numerous vitamins on callogenesis of cowpea and found that there was no effect on growth except with the vitamin nicotinic acid,

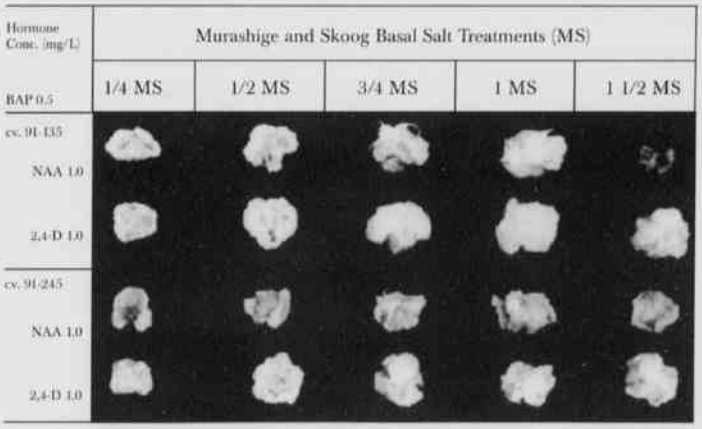

Fig. 7. Callogenesis of cowpea, cv. Early Scarlet (formerly breeding line 91-135) and 91-245, leaf disk explants after 3 weeks in culture with various levels of MS and two auxin types (2,4-D or NAA).

which increased callus production for the cultivar Akadane Onaga.

Effect of Iron on Callus Induction.--Genotype had a significant influence on callus induction, whereas auxin type or the level of iron had no significant effect. Overall, Early Scarlet produced a greater amount of callus (mean = 0.27 g) than 91-245 (callus mean = 0.15 g) (data not shown).

The genetic make-up of a plant has been shown to have perhaps the greatest influence on the in vitro growth of plants (Pierik, 1993; Finer, 1994). Data presented in this study verifies that the plant genotype is the single most important factor influencing the growth of cowpea shoot tip and leaf disk explants. This genotype specific response has also been documented in *Vigna radiata* (Gulati and Jaiwal, 1990) and *Vigna aconitifolia* (Eapen and Gill, 1986).

Conclusions

Plant genotype exhibited a major influence on growth of cowpea explants cultured in vitro. The cultivar Early Scarlet displayed greater growth of shoot tips and greater callus proliferation from leaf disks than 91-245 in these experiments. In our study, full strength MS provided good growth from shoot tips for both cultivars. However, increasing the MS level to 1.5 times the standard concentration induced taller shoots for both of these cowpea genotypes. At this level of MS other growth factors may also be increased dependent on the plant genotype. Iron content of the medium did not have a major effect on quantitative growth of cultured shoot tips, but symptoms of iron deficiency were visible with low levels of iron. Varying the vitamin level in the medium also did not have a significant effect on shoot tip growth or callus induction. When utilizing leaf disk explants, the standard concentration of MS is optimal for callus induction with these two genotypes and with both hormones. This study demonstrates that genotype and hormones have the greatest effect on the in vitro responses of cowpea. Therefore, in regeneration studies on U.S. cowpea cultivars, it will be critical to test numerous genotypes and to carefully evaluate different hormones and hormone concentrations. In addition, the results presented here suggest that media components can affect the growth response of cowpea. Optimization of media components will likely be necessary for different cultivars in order to increase regeneration and transformation rates.

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