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AN IN VITRO COTTON CULTURE SYSTEM FOR THE STUDY OF NEMATODE INFECTION

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Abstract

For the first time, continuous culture in liquid medium of excised roots of Gossypium hirsutum has been achieved, healthy roots being maintained for at least 4 weeks. Cultures were incubated in a minimal rocking condition in the dark at room temperature. Half-strength MS basal liquid medium plus 50 mg myo-inositol, 0.5 mg Nicotinic acid, 0.5 mg Pyridoxine-HCl, 0.5 mg Thiamine-HCl, and 15 g/L glucose was found to be the best medium [CRCM - Cotton Root Culture Medium] for the culture of excised roots.The elongation of roots was more effective in liquid than on agar medium. Root cultures showed differences with various species and medium. However, the pattern of elongation of root tips of cotton was similar: at the beginning, there was a big surge of root elongation, and then a dramatic slow down after the second week, continuing without death even after 10 weeks. This disjointed pattern of elongation was also seen in uncut, soil-grown plants. It is hoped this method has potential use for the research of nematode infection.

Introduction

Plant root or hairy root cultures have received considerable attention as a potential system for the production of plant secondary metabolites (Woo et al., 1997). Much research has been performed on the manipulation of the branching pattern in root cultures since branching can influence production of these metabolites (Yun et al., 1992). In recent decades, much attention has been paid to production systems using roots transformed by Agrobacterium rhizogenes, due to the vigorous growth and ability to also produce secondary metabolites (Woo et al., 1997). The application of excised root cultures has also been applied to study the effects of physiological (Wei et al., 1997) and morphological (Izumi et al., 1995) characteristics. Though root culture has been thoroughly studied in the past for many plant species, it has not been used successfully to study reniform infection of cotton.

Plant parasitic nematodes are a serious threat to cotton production in the US Cotton Belt. Reniform nematodes, Rotylenchulus reniformis, are expected to become an even greater threat in the future with their continued spread into uninfected cotton production areas and with the expansion of cotton acreage in the future. However, studies on the infection by and resistance to reniform nematodes are difficult to perform in vivo in the field, where the active sites of the root may be missed during the extraction of nematode from soil sample. As an alternative, root cultures in vitro could be used as a model to study plant tolerance and susceptibility to the nematode, since the reproduction of those responses in vitro could provide an efficient method to perform exact investigations. As cotton excised root culture in vitro has not been studied in earlier investigations, the present study reported here focuses on the patterns of elongation for the continuous in vitro culture of excised cotton roots.

Materials and Methods

Seeds of five cultivars of Gossypium hisutum (provided by DPL and Dr. E. Cebert, Alabama A&M University) were delinted and then shaken gently in a 2% Tween-20 solution. Subsequently, seeds were sterilized by stirring in a 10% H2O2 solution, and then rinsed one time in 75 ml 70% ethanol and three more times in sterile water. Several seeds were placed onto a Petri dish with water-agar medium, and incubated in the dark for 36-48 hours at 28-32 °C.

The seminal root tips (Fig. 1A) were excised and transferred aseptically into Phytatray IITM Cell Boxes (Sigma) containing sterile nutrient medium. Different nutrient medium formulations, at different strengths of basal MS (Murashige & Skoog, 1962) salts, variable concentrations of glucose and vitamins (Myo-inositol, Nicotinic acid, Pyridoxine-HCl, Thiamine-HCl), with and without different concentrations of auxin (NAA), cytokinin (kinetin) and antioxidizers, etc. (PVP-40, Abscisic acid, 2-Mercaptoethanol) were tried. Media were adjusted to pH 5.8 and autoclaved. The roots were grown in the dark at 25°C on an oscillating table, rotated at 50 rpm (Fig. 1B). At 7-day intervals for 4 weeks, growth in length was measured, and these cultured roots were transferred to fresh medium or root tips of the main axis were excised and subcultured. Five to thirty replications, each of which contained two or three root samples, were established and the experiment was repeated twice. The ANOVA was analyzed as repeated measures by SSPS software.

Results and Analysis

Optimization of Continuous Excised Root Culture

A typical composition for a root culture medium contains mineral and organic salts, vitamins, carbohydrate, hormone(s) and antioxidizer(s). On the strength of basal MS salts, variable concentration of glucose and vitamins (Myo-inositol, Nicotinic acid, Pyridoxine, Thiamine), six media compositions (presented in the table of Fig. 2.) were made. DPL5415 root tips were cut 4 cm long from 2 days seedlings and keep continuously in culture within the following 4 weeks. Time course of root elongation showed differences with the various compositions (Fig. 2). The actively growing roots, that initially looked healthy and white in color, then decreased in number, the response varying with the treatment. Amongst the different combinations tried, CRCM (Cotton Root Culture Medium) was found to be the best Medium. CRCM1 and CRCM 3, had similar patterns of root tip elongation with CRCM: at the beginning, root tips became swollen and a notable length increment (a bigger "spurt") was found for the root elongation, and then a slow down after the second week, but which kept growing continuously (Fig. 6B, C, D). Comparison of CRCM with CRCM2, half-dilution of MS salts sustained better root growth than Full Strength. Increasing glucose concentration, from CRCM to CRCM3, decreased root elongation. Increasing vitamin concentration did not improve root elongation, especially for Thiamine. The elongation of roots was more effective in liquid than on agar medium. Under normal conditions, liquid medium was better for the growth rate of root, number of branch roots, and elongation of the root per unit time than agar media. The addition of auxin (NAA), cytokinin (kinetin) or antioxidizer (PVP-40, Abscisic acid, 2-Mercaptoethanol) did not any increase root elongation or root viability in the present research. If the concentration was too great, almost no root growth was observed and root died eventually.

Comparison of Different Excision Methods

Using DPL 5415 root tips, research on the method of excision was conducted. During continuous culture in CRCM, the comparison of different initial cutting length is presented in Fig. 3A. Both initial length treatments had the same developmental pattern of root tip elongation. However, the initial root tip length of 4 cm was better than 1cm in overall root elongation. Figure 3B compares the growth of 1 cm- and 4 cm-long initial root tips over different culture periods. These root tips were excised weekly and subcultured to fresh medium. Again, they presented the same overall growth pattern and the initial root tip length of 4 cm was also better than 1cm in root elongation (Fig. 3B). If the initial root tip length was the same, the excision length of 4 cm was better than 1 cm in root elongation, suggesting that a longer piece of root tip was required to maintain fast elongation.

Root Elongation Pattern for the Different Varieties and Environments

When continuously cultured in CRCM with a 4 cm long initial tip, we observed a genotype-independent effect on the pattern of root elongation (Fig. 4A, B). Roots kept growing, even after 10 weeks. The maximum actual lengths of root were 38 and 95 cm, after 1 week and 4-week culture, respectively. The average elongation length of these five varieties were 16.45, 7,76, 3.98 and 1.65 cm from the first to the fourth week, respectively. DPL 5415 and DPL 429 were the best varieties for continuous excision root culture. However, the pattern of elongation of excised root tips of cotton was similar: at the beginning, there was a bigger spurt, and then a slow down after the second week.

The rate of root elongation is much faster during the first week than in the fourth week. After observing root growth in pots placed in the same room, we found the same pattern with root culture using CRCM under sterile conditions (Fig. 5). Excised root in vitro culture was much faster in elongation than natural root growth (Fig. 5A, Fig. 6A), and there was a higher percentage of the total growth during the first week (Fig 5B). Although both of the culture methods have same pattern of root elongation, the elongation curve is flatter for the natural root growth method (Fig. 5A).

Discussion

Early in 1938, there was an attempt to grow excised roots of 30 species of dicotyledonous plants in vitro (White, 1938). Moreover, there has been no previous study on cotton, up to now. The data presented here provide a suitable method for the in vitro growth of excised roots of cotton with a number of different varieties, and makes it possible to obtain an accurate report for in vitro cotton root research. Since the culture conditions tested here allowed the root to grow, but at different rates, depending on the treatments and on the genotype, excised root culture could be used as an experimental system to test the effect of different biotic and biotic factors. This suggests that the excised root culture described here could be used as a model to evaluate nematode infection in future.

We found that cotton roots did not require a high concentration of MS salts, vitamins and glucose supplements to grow in the dark, which was different from Marin (1998) on Prunus rootstocks. A decrease in root elongation rate was observed in most of the roots after the first week of culture. Such a reduction was also seen in natural (uncut) root growth. The difference in curve shapes may be either a reduction in the quantity of metabolites supplied to the root apex or a qualitative change in the composition, causing inhibition of cell division after removal of the mother tissue. Although Macdonald (1982) had a similar

report, the research on the time course of in vitro root elongation culture is still sporadic. Thus, the exact reason for the results of the present investigation requires further study.

Acknowledgements

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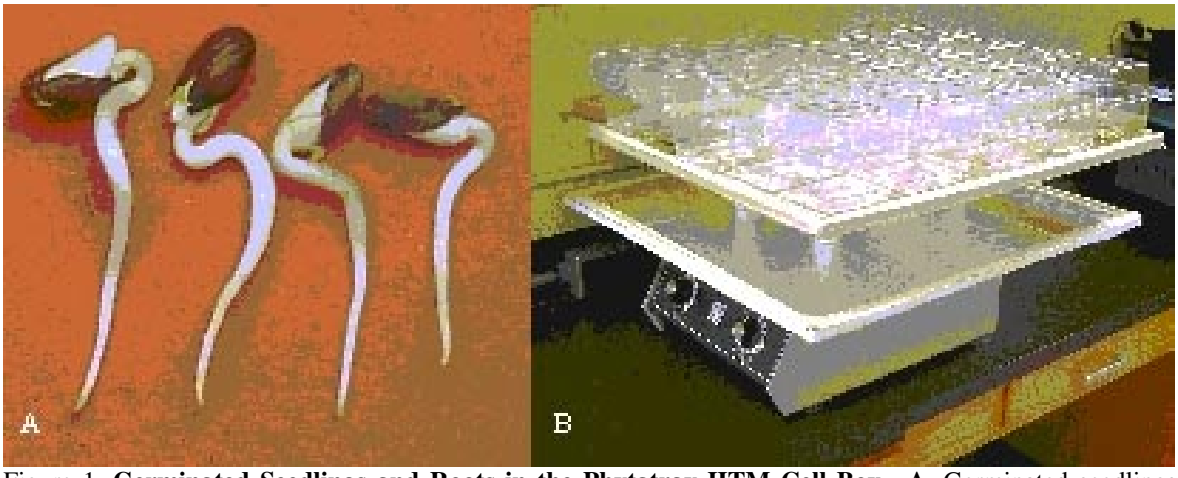
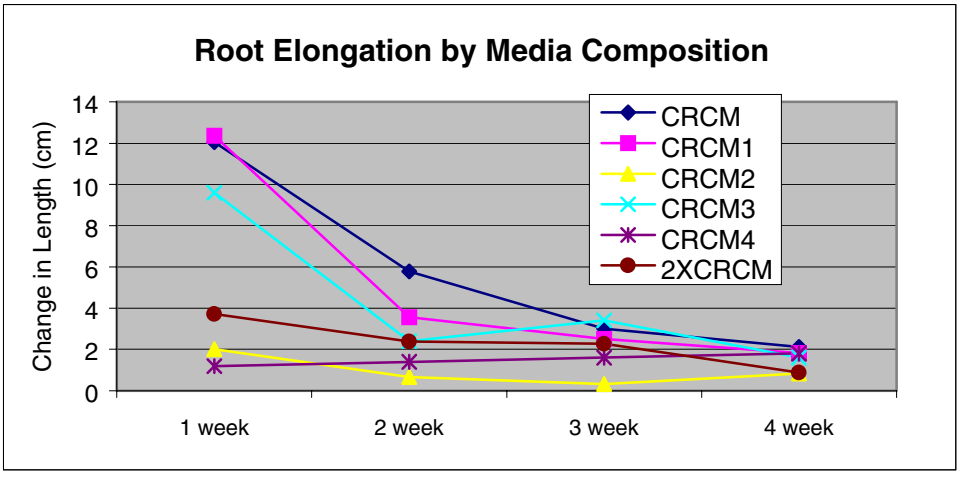


Figure 1. **Germinated Seedlings and Roots in the Phytatray IITM Cell Box**. **A.** Germinated seedlings with seminal root tips. **B.** Excised root tips were transferred into a Phytatray IITM Sigma Cell Box containing sterile nutrient medium, and were grown in the dark at 25°C, rotated at 50 rpm.



Medium	MS Salts	Glucose 15 g/L	Myo-inositol 50 mg/L Nicotinic acid 0.5 mg/L Pyridoxine 0.5 mg/L	Thiamine 0.5 mg /L
CRCM	0.5X	1X	1X	1X
CRCM1	0.5X	1X	2X	20X
CRCM2	1.0X	1X	1X	1X
CRCM3	0.5X	2X	1X	1X
CRCM4	0.5X	1X	1X	20X
2xCRCM	1.0X	2X	2X	2X

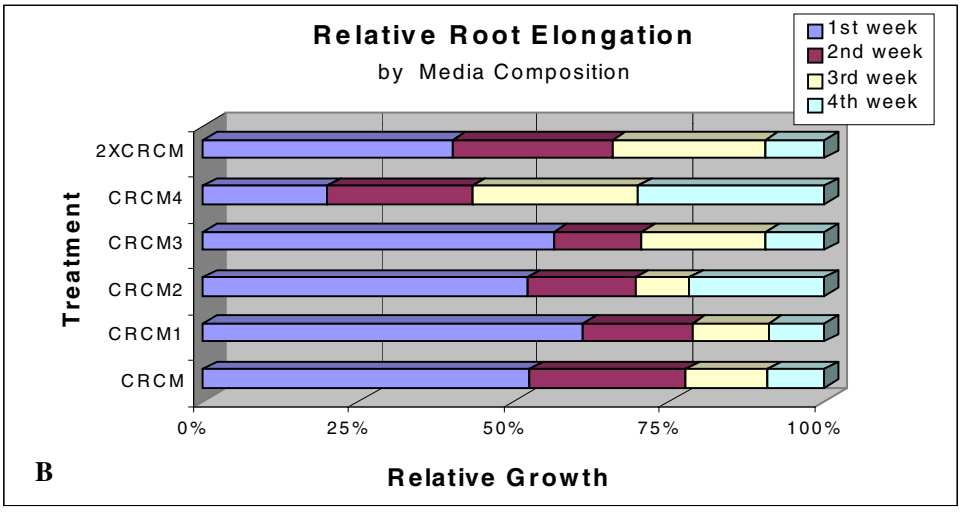


Figure 2. **Timecourse of Six Media Compositions on Root Elongation of Cotton Variety DPL 5415. A.** Change in weekly root length for six media compositions. **B.** Relative root elongation for six media compositions. ANOVA analysis showed there was significance ($P<0.01$) among the six media compositions.

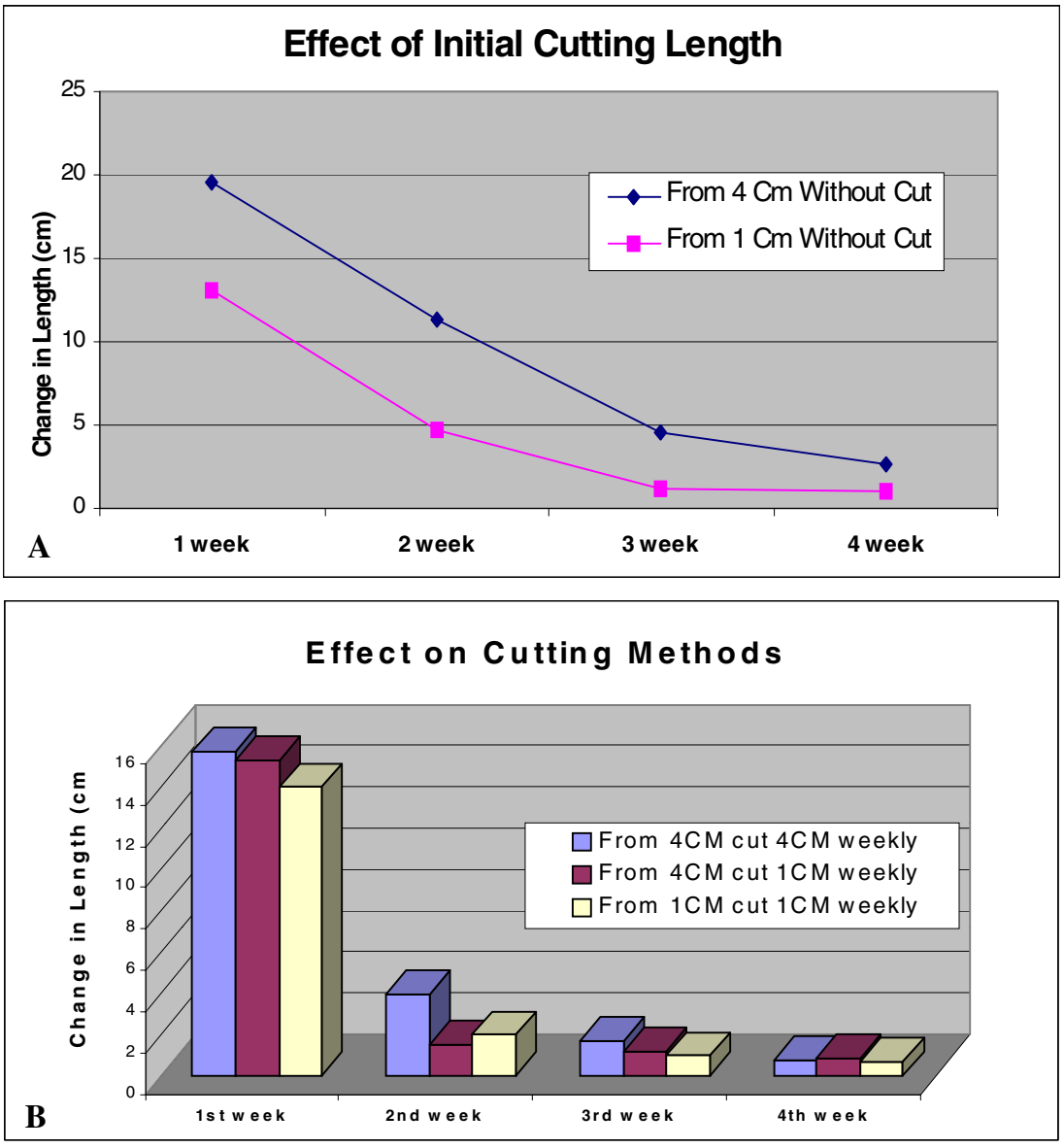
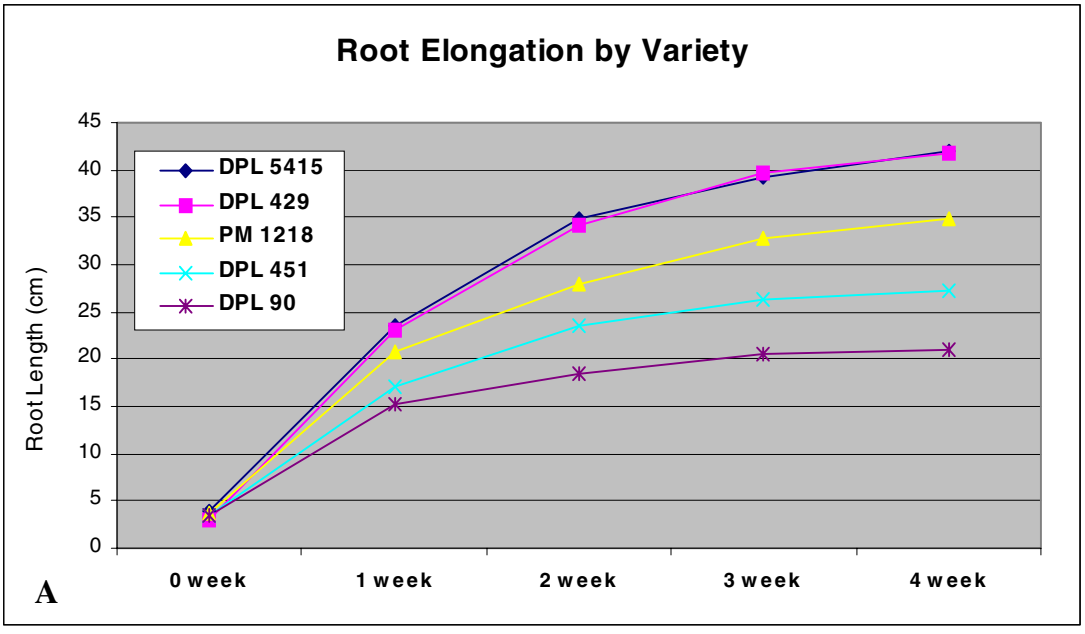


Figure 3. **Timecourse of Different Initial Cutting Length and Excision Method for Variety DPL 5415. A.** Change in weekly root length for different initial cutting length. **B.** Change in weekly root length for different cutting method.



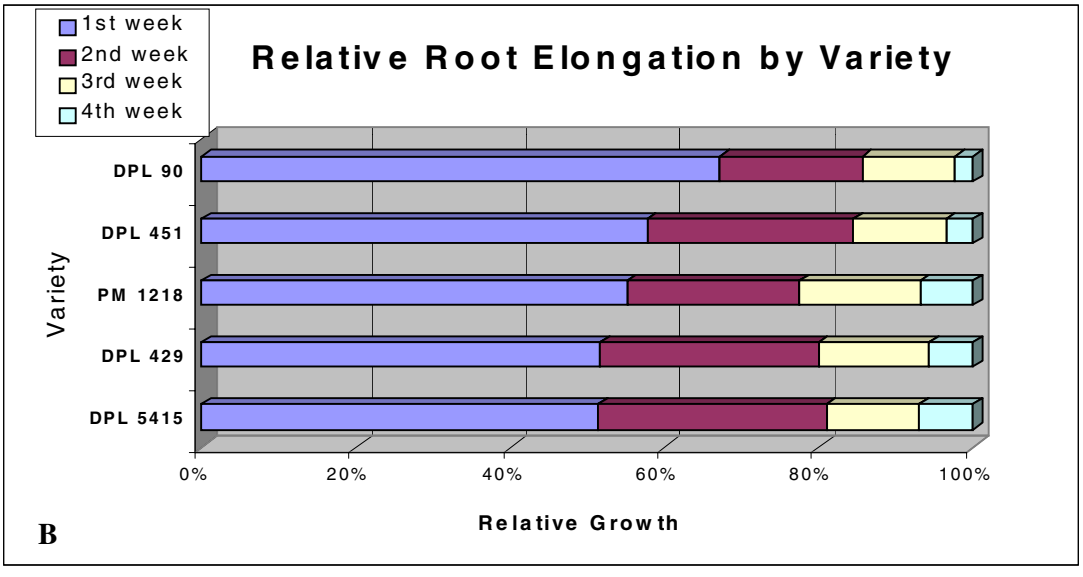
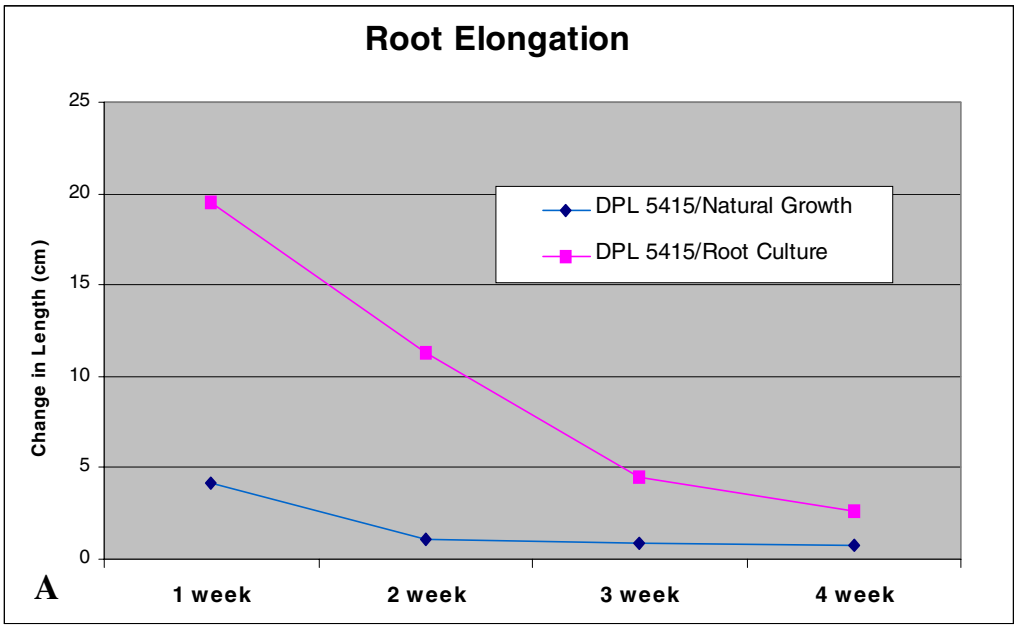


Figure 4. **Timecourse of Root Elongation for Different Varieties. A.** Change in weekly root length for different varieties. **B.** Relative change in weekly root elongation for different varieties. ANOVA analysis showed there was significance ($P<0.01$) among the five varieties.



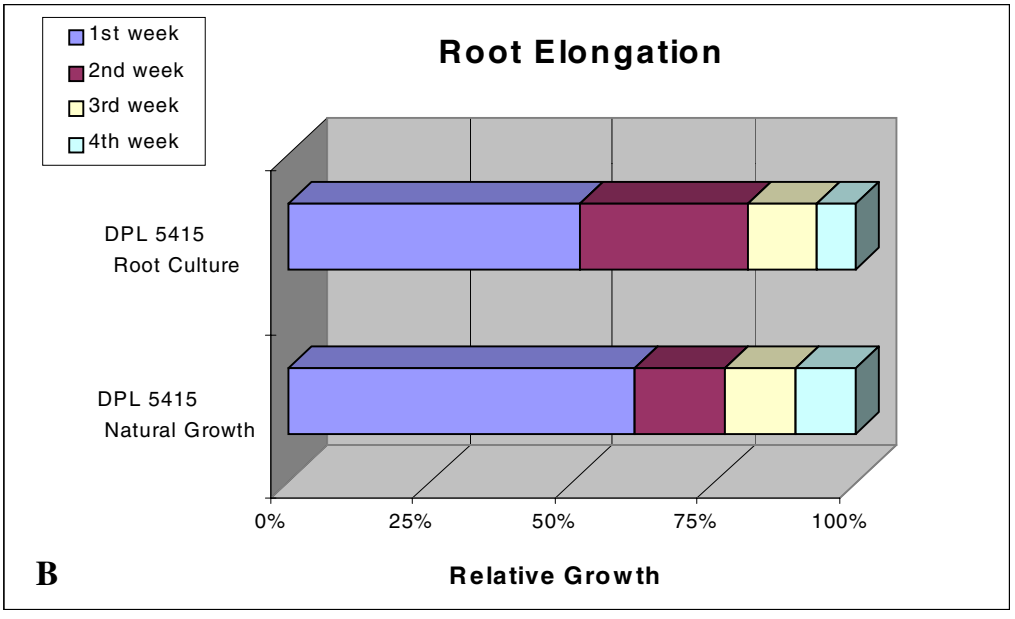


Figure 5. **Timecourse of Root Elongation for Different Culture Treatment.** **A.** Change in weekly root length for different treatments. **B.** Relative change in weekly root elongation for different treatments. ANOVA analysis showed there was significance ($P<0.01$) between the two treatments.

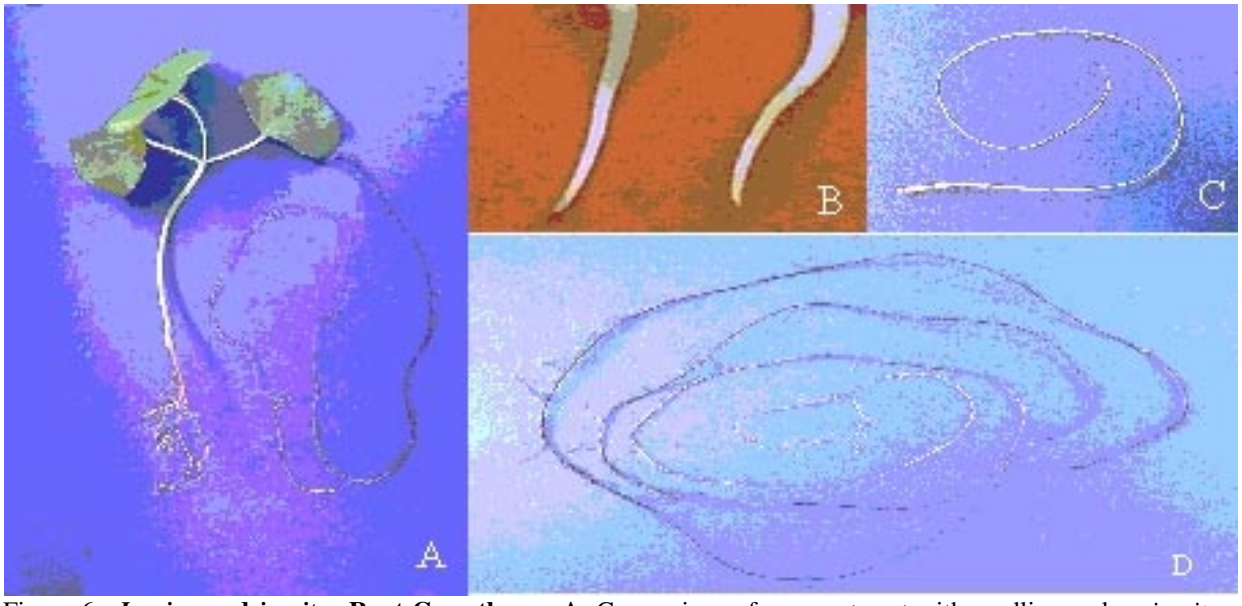


Figure 6. ***In vivo* and *in vitro* Root Growth.** **A.** Comparison of an uncut root with seedling and an *in vitro*-cultured root. **B.** The seminal root tips before *in vitro* culture. C. An *in vitro* root cultured 1 week. D. An *in vitro* root cultured 4 weeks.