Initiation of Callus in Cashew nut, *Anacardium occidentale* L. from Plumular – and Cotyledonary Tissue Excised from Mature Nuts

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Abstract
The aim of the study was to determine the optimal cultural conditions for the initiation of callus in plumular –and cotyledonary tissue excised from mature cashew nuts. The explants derived from mature cashew nuts were screened at 4 concentration combinations of BAP and 2,4-D in the range 0-50mg l^{-1} for callus production. Within the main treatment combinations of 4 levels of acid-washed activated charcoal, in the range 0-0.5% w/v, were included as sub-treatments.
Responses extended from swelling of embryos and cotyledons to the proliferation of adventitious roots and white callus-like structures. A number of combinations produced abnormal plants with curled leaves and multiple shoots.

Keywords: somatic embryogenesis, zygotic embryo, activated charcoal, auxin, cytokinin

1 Introduction

1.1 World Production and Importance of Cashewnuts

World production of cashew nuts grew rapidly during the 1950s, reaching peak production of 510, 000 metric tonnes of raw nuts in 1974 (Jaffer, 1994). The bulk of this production came from three countries, namely India, Mozambique and Tanzania (Behrens, 1996). However, starting from 1975, and continuing through the mid-1980s, there was a drastic decline in global production of cashew nuts, which led to a very constricted international market. According to Jaffer (1994), the cause of this global decline may have been related to the reduction of cashew production in Mozambique and Tanzania. Since the mid-1980s, there has been some recovery in world production, primarily, due to a significant expansion in Brazilian production. In the late 1970s, of the five major edible nut crops produced world-wide, the cashew ranked third after almonds and walnuts (Ohler, 1979). Current world production of cashew nuts is estimated at 788, 231 metric tonnes with the major contribution (over 70%) coming from leading producers: Brazil, India, Vietnam, Tanzania, Indonesia, and Mozambique.

The cashew is native to northeastern Brazil. It spread to parts of South and Central America. In the 16th century Portuguese colonists and missionaries introduced it to India and East Africa (Verheij and Coronel, 1991). It was taken to India and East Africa mainly as an exotic tree crop which could grow on poor soils but it also proved useful for soil erosion control.

1.2 Botany of the Cashewnut tree

The cashew tree, *Anacardium occidentale* L., belongs to the genus *Anacardium* and is a member of the family, Anacardiaceae. Other important fruits and nuts, such as the mango (*Mangifera indica* L.) and the pistachio nut (*Pistachia vera* L.) belong to this family (Ohler, 1979). The cashew is a woody, evergreen tree with an umbrella-like spreading habit and grows to a height of up to 10-15 metres. Large alternate dark green leaves are borne on long branches. Young leaves are reddish-brown to green, gradually turning to dark green as they mature. At the end of the branches, flowers are produced in loose panicles.

The cashew inflorescence is andromonecious; on each panicle perfect flowers and staminate flowers are produced (Behrens, 1996). The crop is cross-pollinated to a large extent. Normally trees growing under favourable conditions may produce their first crop worth harvest at the age of three years. The kidney-shaped nut is attached on the fruit-like pear-shaped, cashew 'apple' which is an enlarged and swollen fruit stalk (Verheij and Coronel, 1991). The true fruit is the nut with a hard, greyish-brown pericarp. It consists of two cotyledons and the embryonic axes. The root system of the cashew tree includes an extensive lateral root system and a taproot that penetrates deeply into the soil (Gibbon and Pain, 1985).

Cashew is a hardy tropical tree (Purseglove, 1977) and it can tolerate a wide range of environmental conditions. It grows well on a variety of soils and will survive with as little as 750mm of rain per annum. The cashew has a well-developed and fast growing root system with a network of primary and secondary roots providing the tree with its mineral requirements in poor soils and water in seasons of drought. Being a tropical plant, cashew thrives at high temperatures. In its native habitat it extends into the semi-arid regions where daily maximum temperatures may exceed 40^0C, for example, in Northern Mozambique.

1.3 Tissue Culture and Micropropagation in the Cashewnut

There are many technical problems associated with propagation of cashew through conventional methods. Better methods of raising clones from existing elite cashews, or from F_1 hybrids would provide solutions to limitations inherent in conventional propagation (Philip, 1984). *In vitro* culture or tissue culture in cashew could offer an efficient, rapid and, possibly, a cost-effective system, which could be used, particularly for multiplying large numbers of self-rooted selected plantlets of elite cashew genotypes for breeding and production purposes.

Techniques of *in vitro* multiplication of cashew through micro-propagation via axillary branching and *in vitro* organogenesis and embryogenesis have been attempted in the past. Cashew, like other members in the family Anacardiaceae (pistachio or mango), has been shown to be strongly recalcitrant to *in vitro* techniques, and only limited successes have been achieved as yet in most of the tissue culture work that has been done in cashew.

Attempts by Jha (1988) to induce somatic embryogenesis in cashew resulted in the formation of callus and globular protuberances in 65% of cases on immature whole embryo-like structures which had many aberrations and misshapen forms. In addition, calli of different cashew explant origins have been reported to produce roots (Falcone and Leva, 1987). Sy *et*

al (1991) obtained a mass of white "embryoids" with no organised structure (2%) frequency after testing the ability of different cashew explants derived from cotyledons, leaves and petioles to induce somatic embryogenesis, organogenesis or direct regeneration through callus. Normal somatic embryos were not observed. Although direct somatic embryogenesis from mature and immature cotyledon sections has been reported (Hegde *et al*., 1990, 1991), a protocol for large-scale production of somatic embryos in cashew has not yet been established. However, the predominant use of zygotic embryo-derived explants with unpredictable genetic potentials presents little prospect for their more practical use in rapid clonal propagation.

Some degree of progress with the application of micropropagation in cashew has been achieved. Micropropagation via organogenesis has been used by many investigators (Falcone and Leva, 1987; 1989; Lievens *et al*., 1989; Leva and Falcone, 1990; Mantell *et al*., 1998) to induce shoot elongation on microcuttings from seedlings and bud growth on cotyledons from mature seed and young leaves from seedlings. Das *et al.* (1996) used nodal cuttings and shoot tips, and cotyledonary nodes from *in vitro*-raised seedlings were used by other investigators (D'Souza and D'Silva, 1992; Das *et al*., 1996). Despite these successes with application of micropropagation in cashew some problems with *in vitro* explant viability, consistency in bud sprouting and shoot elongation still exist. Other difficulties remain to be resolved in the multiplication of cashew microshoots. During their studies all the investigators involved with experiments on micropropagation of cashew reported that *in vitro* rooting and survival of *in vitro*-produced plants of cashew were constraints to more widespread application of micropropagation techniques to cashew. The latter investigators (D'Souza and D'Silva, 1992; Das *et al*., 1996) increased the rooting percentage of cashew microshoots up to 80-90% by immersing the shoot bases in a suspension of wild *Agrobacterium rhizogenes* (LBA 9402). However, the rate of survival of the treated plantlets *ex vitro* never exceeded 28%.

Recent studies undertaken by Boggetti (1997) on factors affecting shoot development from nodes, *in vitro* root induction from various explant types and *de novo* regeneration of shoots and somatic embryos from immature cotyledon explants and Mantell *et al* (1998) on micrografting in combination with micropropagation have given promising results. Rooted microshoots were successfully weaned in the glasshouse and regeneration of embryoids was achieved from immature cotyledon explants. The embryoids developed further into secondary embryoids and leaf-like structures retaining their morphogenetic potential even after two years. Micrografting shoot tips by a modified side grafting procedure on *in vitro*-raised seedling rootstocks had micrografting success rates in the range 40-80%.

Tissue culture techniques can be used for long-term storage of germplasm. Other potential applications of tissue culture techniques to cashew include accelerated screening programmes for disease resistance and development of disease-resistant cultivars (particularly for anthracnose and powdery mildew). Integration of foreign genes through bacterial vector-mediated transfers now being developed for other fruit species, using *in vitro* culture, may also have future applications for cashew. Micrografting in combination with micropropagation have given promising results and these results could have implications in relation to safe international transfers of cashew germplasm in the future.

2 Materials and Methods

The plumule is a source of juvenile tissue and it is expected that such tissue will respond more readily and rapidly to tissue culture than most explant types already investigated (Chan *et al.*, 1998). The major aim of the study was to explore the possibility of developing a simple but efficient method of optimising cultural conditions necessary for the induction of callus using plumular tissue excised from mature zygotic embryos of cashew as explants, and comparing with the response of cotyledonary tissue. The experiments described below were carried out in the Unit for Advanced Propagation Systems (UAPS).

2.1 Experimental Design

Different combinations of concentrations of plant growth regulators, 2,4-D and BAP and the role of activated charcoal (AC) were assessed to determine the most optimal cultural conditions for callus induction and subsequent growth and development of plumular tissue excised from mature zygotic embryos, and cotyledonary tissue. The experiment was set in a completely randomised design in a 4 x 4 x 4 factorial arrangement with 5 replicates for each explant type. The treatments were derived from the combinations of the levels of each factor.

2.2 Plant Materials

The plant materials used in all studies in this project were derived from the cashew nut tree, *Anacardium occidentale* L. The explants used in the project were plumular tissue excised from mature zygotic embryos and cotyledonary tissue from mature cashew nuts. The nuts were obtained from the mother stock trees growing at the Agricultural Research Institute (ARI), Naliendele in Tanzania. Accession AC 10, a selected clone of cashew originating from Sri Lanka, was used in all the *in vitro* culture work done in this study.

2.3 Composition and Preparation of Growth Media

Full strength modified Murashige and Skoog (1962) basal salts media which were supplemented with sucrose at 3% were used. Plant growth regulators, BAP and 2, 4-D, were prepared as stock solutions and added to the media (at range of 0-50 mg l^{-1}) at the time of preparation. Prior to addition of the gelling agent and activated charcoal, the pH of the medium was adjusted to 5.8 and then boiled. The gelling agent used was phytagel at 0.3% w/v. Acid-washed activated charcoal (AC) was added to the medium (at range 0-0.5% w/v). The medium was then dispensed as 10ml aliquots into 25mm x 75mm flat-bottomed soda-glass culture tubes using pipettes and/or syringes. All media prepared were sterilised for 20 min at 120^0C at 103.5 kPa pressure in an autoclave. Autoclaved media were then stored for 4-5 days before explant inoculation or culture transfers.

2.4 Surface Sterilisation of Explants

For optimal results, mature cashew nuts were surface sterilised according to the procedure described by Mneney, (1998). Mature nuts were left in concentrated sulphuric acid (98%) for 16 hours and washed in running water until the oxidised slurry deposit was completely removed. The mature nuts were further subjected to a standard sterilisation treatment using bleach.

2.5 Excision and Culture of Tissue Explants

Mature nuts were cut longitudinally into two halves in order to extract two sections of cotyledonary tissue with the embryonic axes seated in the upper portion of the nuts. The embryos each bearing the radicle and plumule were excised by separating the cotyledons of the nuts. Following excision from the cotyledons, an embryo and one of the cotyledonary halves were cultured intact and separately into 25mm x 75mm flat bottomed soda-glass culture tubes on gelled MS salts medium.

2.6 Culture Incubation and Subculturing

Cultures were incubated, exclusively, in one of the UAPS growth rooms at a stable temperature of 27^0C ± 0.5^0C under dark conditions for 4 weeks before the first subculture. Subculturing was done regularly every 4 four weeks until callus initiation. Tissues were transferred, as they were to fresh medium similar to the initiating medium and were incubated under similar light and temperature regimes prior to subculturing.

3 Results

This report highlights results obtained from a study in which activated charcoal was tested in association with two growth regulators, auxin and cytokinin, 2, 4-D and BAP, respectively. The different variables that were recorded were the development of callus on the explants, elongation of the explants, development of adventitious roots and browning of the growth medium. Multiple shoots and abnormal plantlets were also seen developing on tissue in some of the treatments.

3.1 Evaluation of 2, 4-D, BAP and activated charcoal on the development of callus at 2 and 6 weeks.

There was no sign of callus in any of the treatments after 2 weeks of incubation. Six weeks of incubating the cultures produced random and isolated occurrence of callus-like structures developing on plumular and cotyledonary tissue. At 50 mg l^{-1} 2, 4-D + 0 mg l^{-1} BAP with AC at 1 g l^{-1} plumular tissue showed brown callus-like structures. Activated charcoal at 1 g l^{-1} in association with 2, 4-D at 50 mg l^{-1} + 10 mg l^{-1} BAP, produced callus-like structures on both types of explant. 2, 4-D at 25 mg l^{-1} + BAP at 25 mgl^{-1} and 2, 4-D at 50 mg l^{-1} + BAP at 50 mg l^{-1} both at 1 g l^{-1} AC showed development of callus-like structures only on cotyledonary tissue.

3.2. Evaluation of activated charcoal, 2, 4-D and BAP on the formation of adventitious roots on plumular and cotyledonary tissue at 2 weeks.

There were no significant differences where AC + 2, 4-D and AC + BAP influenced adventitious root formation on plumular and cotyledonary tissue among all the treatments. Figures 1a and 1b show that there were no significant differences in root formation on plumular and cotyledonary tissue due to 2, 4-D and BAP across all treatments.

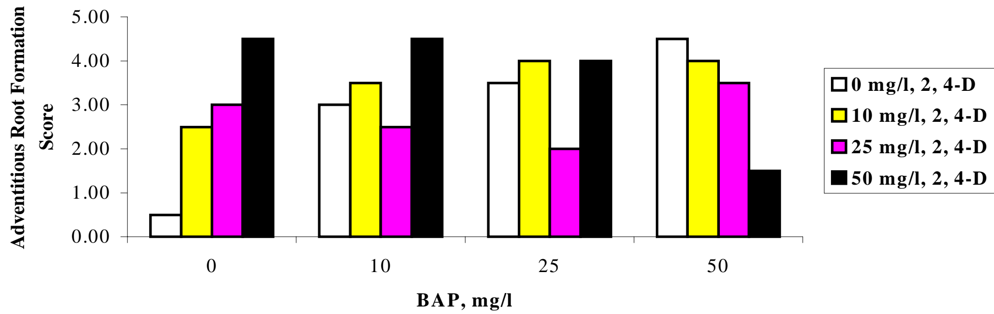

Fig 1a. Mean effects of 2, 4-D and BAP on adventitious root formation on plumular tissue at 2 weeks; n = 5, lsd (p = 0.05) = 1.75.

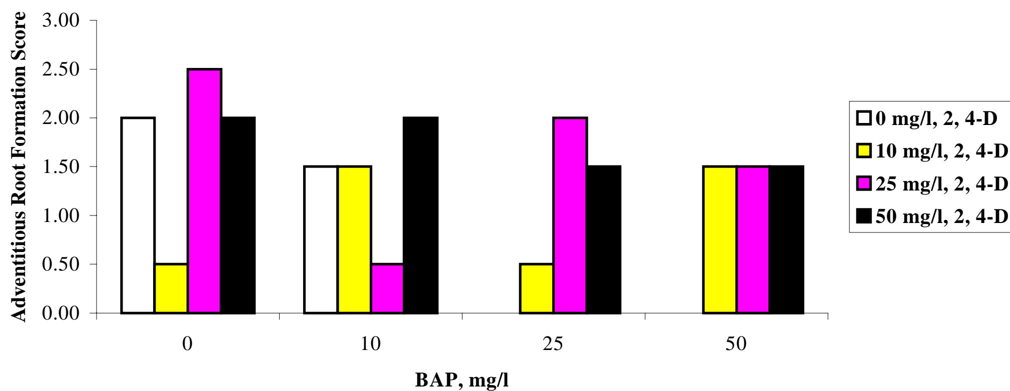

Fig 1b. Mean effects of 2, 4-D and BAP on adventitious root formation on cotyledonary tissue at 2 weeks; n = 5, lsd (p = 0.05) = 2.33

3.3 Evaluation of and activated charcoal, 2, 4-D and BAP on the formation of adventitious roots on plumular tissue and cotyledonary tissue at 6 weeks.

At 6 weeks there was a similar trend as at 2 weeks on root development on plumular and cotyledonary tissue as there were no differences due to AC and 2, 4-D within the treatments. Although AC at 50 g l^{-1} + 50 mg l^{-1} BAP showed the highest response there were no significant differences on the responses of plumular tissue to root formation (Fig 2a). Cotyledonary tissue showed a gradual increase at 2.5g l^{-1} AC reaching a maximum at 50 mg l^{-1} BAP (Fig 2b).

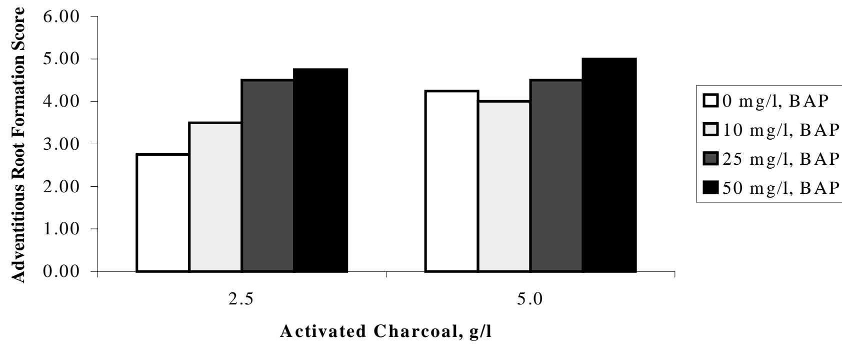

Fig 2a. Mean effects of AC and BAP on adventitious root formation on plumular tissue at 6 weeks; n = 5, lsd (p = 0.05) = 1.28

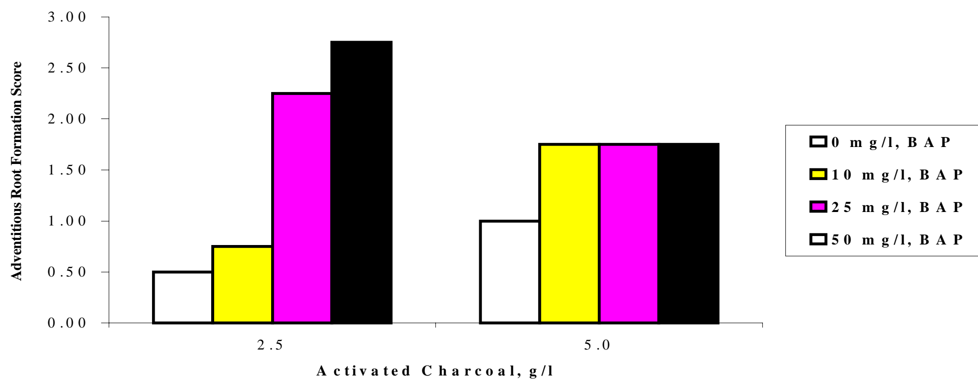

Fig 2b. Mean effects of AC and BAP on adventitious root formation on cotyledonary tissue at 6 weeks; n = 5, lsd (p = 0.05) = 1.94

There were no significant differences in the mean scores for both plumular and cotyledonary tissue in terms of root formation. However, various patterns of root development were observed within the treatments. Whereas plumular tissue tended to result in increased rooting at increasing 2, 4-D and BAP levels, the magnitude of rooting at the same levels was much smaller for cotyledonary tissue.

Adventitious root formation on cotyledonary tissue at 6 weeks was greatest at 2.5 g l^{-1} AC + 50 mgl^{-1} BAP followed by 2.5 g l^{-1} AC + 25 mg l^{-1} BAP. At 5 g l^{-1} AC the differences were not significant.

Auxin and cytokinin interactions gave comparatively higher responses on plumular tissue at combinations of 50 mg l^{-1} 2, 4-D with all concentrations of BAP (Fig 3a). Smaller responses with decreasing concentrations of 2, 4-D with all concentrations of BAP were also observed. There was a gradual rise in rooting at 0 mg l^{-1} 2, 4-D, which fell as BAP concentrations increased. Cotyledonary tissue showed varied responses which could not be explained (Fig 3b).

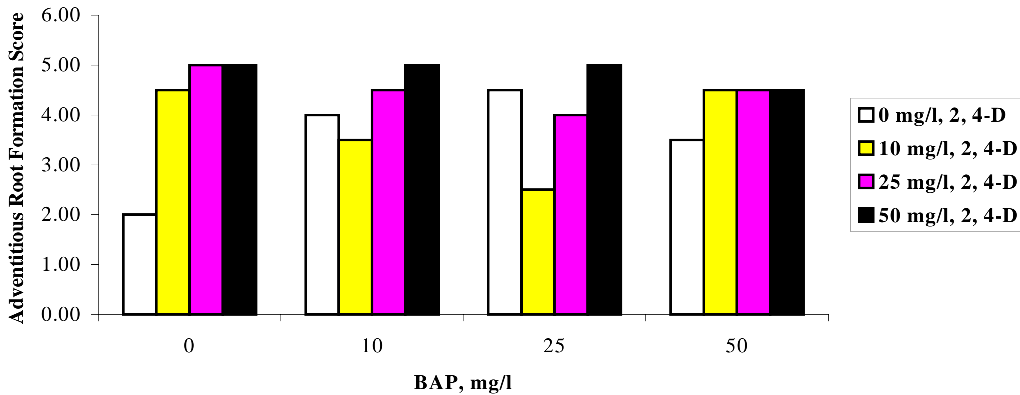

Fig 3a. Mean effects of 2, 4-D and BAP on adventitious root formation on plumular tissue at 6 weeks; n = 5, lsd (p = 0.05) = 1.81

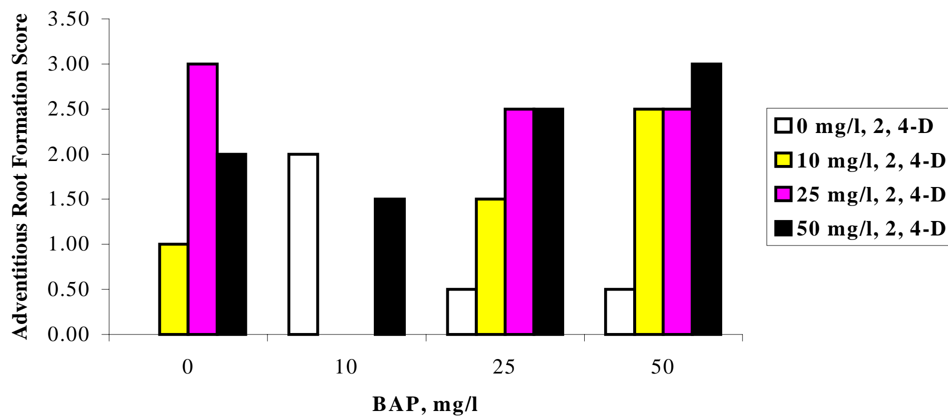

Fig 3b. Mean effects of 2, 4-D and BAP on adventitious root formation on cotyledonary tissue at 6 weeks; n = 5, lsd (p = 0.05) = 2.75

3.4 Development of Multiple Shoots and Abnormal Plantlets

Multiple shoots and abnormal plantlets were observed developing on cotyledonary tissue and plumular tissue, respectively. At 0 mg l^{-1} 2, 4-D + 25 mg l^{-1} BAP, multiple shoots developed in medium with 1, 2.5 and 5 g l^{-1} AC. Abnormal plantlets developed on plumular tissue in medium with 1 g l^{-1} AC and combinations 10 mg l^{-1} 2, 4-D and 10-25 mg l^{-1} BAP.

4 Discussion and Conclusions

It has widely been recognised that auxins play an important role in callus induction as the initial stage in somatic embryogenesis. Auxins are considered the main agents for tissue dedifferentiation, which often results in callus formation. One of the auxins that have shown to have a close relationship to somatic embryogenesis is 2, 4-D.

In the case of cashew the amounts of AC, 2, 4-D and BAP show some interactions. The results derived from the project seem to indicate that at 1g l^{-1}, AC had a significant influence

on the development of the callus-like structures on plumular and cotyledonary tissue. It also appears that callus-like structures formed at high levels of auxin (25-50mg l^{-1}) with or without cytokinin. The cytokinin at all concentrations interacted with the auxin at 0, 25 and 50mg l^{-1} to induce callus on the tissues. At low concentrations of BAP (0-10 mg l^{-1}) callus-like structures were produced on plumular tissue and to some extent on cotyledonary tissue. Higher BAP levels, 25-50 mg l^{-1}, resulted in development of callus-like structures on cotyledonary tissue only. This could have been due to different levels of tolerance by the explant types to the 2, 4-D and BAP. Younger tissue is normally more tolerant than older tissue. Another reason could have been the effect of 2, 4-D in the presence of BAP.

Adventitious root formation on both types of explant at 2 weeks showed no significant differences but it followed an interesting pattern. Root formation on plumular tissue formation increased with lower 2, 4-D concentrations and 0-50 mg l^{-1} BAP. Higher 2, 4-D levels and all BAP concentrations led to reduced root formation. A similar pattern was observed at 6 weeks for plumular tissue where root formation without 2, 4-D increased with increasing BAP. Higher 2, 4-D levels gradually reduced the formation of roots as cytokinin levels also increased from 10-25 mg l^{-1} but up to 25 mg l^{-1} BAP. At all levels 2, 4-D seemed to stabilise at 50mg l^{-1} BAP. The best response was obtained from 50 mg l^{-1} 2, 4-D at all BAP levels. A similar trend as at 2 weeks seemed to follow during the 6th week of incubation. Root formation also increased with increasing levels of BAP at both AC levels. Because cotyledonary tissue followed no logical pattern the findings were not conclusive.

Because of the nature of the experiment the type and size of culture vessels could have had an influence on the results. Therefore, in future investigations there will be need to consider the option of using bigger vessels with increased volume of medium while at the same time reducing the size of the explants especially the cotyledonary tissue. The work on callus initiation was successful but not conclusive. More investigations need to be carried out in order to determine whether or not the callus-like structures were "true callus" and methods for regenerating plants through somatic embryogenesis need to be established. In addition, the timing for callus separation from the mother explant requires further attention.

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