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# PRELIMINARY STUDIES ON *in vitro* PROPAGATION OF YAM BEAN (*Pachyrhizus erosus* (L.) Urban)

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## ABSTRACT

The regeneration potential of *Pachyrhizus erosus* using single node culture has been investigated on Murashige and Skoog (1962) (MS) medium alone, and supplemented with each of Benzyladenine (BA), 2-isopentyladenine (2-ip), Kinetin (Kin), Zeatin and Adenine, at a 1 mg/l concentration. The position effect of the nodes was investigated for hormone sensitivity. The basal medium must be supplemented with hormone for bud burst. Buds were stimulated by a range of cytokinins, but response was not dependent on nodal position. BA, Zeatin and Kinetin, however seem to induce multiple bud proliferation. Callus induction using leaf explants of different physiological ages was investigated on MS medium supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D); 3 indoleacetic acid (IAA) plus Kin; 1 naphthylacetic acid (NAA) plus Kin; and 2,4-D plus Kin at 1 mg/L concentration. The effect of light on callus initiation was noted. Callus was initiated on MS with 2,4-D plus Kin, also NAA plus Kin incubated in complete darkness. The quantity of callus varied with leaf age. Callus initiated with 2,4-D and Kinetin was placed on MS supplemented with BA using either fructose or glucose as the carbon/energy source, and incubated in light and dark environments. Chlorophyll formation was apparent in callus exposed to light, when the carbon source was fructose. Squash preparations of callus on BA supplemented medium indicated the presence of tracheids. Plantlet regeneration from callus has not yet been achieved.

## INTRODUCTION

There has been much interest generated recently in the Yam Bean, genus *Pachyrhizus*, family Fabaceae, due to its potential as a root tuber crop in the tropics. The possibility of its cultivation in Third World countries due to its ready adaptability to cultivation in the wide range of tropical climates, and its ability to resist pests has been indicated (Anonymous, 1979). The resistance to pest attack is due to the presence of a chemical substance - rotenone - in the vegetative parts of the plant (Duke, 1981). This substance has known insecticidal properties, and has been widely used in the control of insect pests. The Yam Bean therefore offers a possible partial substitute for rotenone insecticides (Hansberry and Lee, 1943). This interest has led to a complete taxonomic revision of the genus (Sorensen, 1988).

The crop is mainly cultivated, however, for its large root tubers which are of high nutritional value with respect to its protein and starch content. Two edible Yam Beans, Pachyrhizus erosus and P. tuberosus, have been cultivated in Central America, India and China.

The Yam Bean also has the advantage of being a legume, thus providing its nitrogen needs by nitrogen fixation and may even enrich the soil in which it is grown. Under long day conditions it develops aerial parts rich in proteins, which could be used as forage or as green manure (Zinsou et al., 1987a).

Despite all its possible attributes, the Yam Bean still remains a primitive undeveloped crop. Several requirements would have to be met in the development of the Yam Bean as a root tuber crop in the tropics. Large scale production of high quality, high yielding varieties, which are relatively pathogen-free would be required. Comprehensive germplasm collections would also have to be established throughout the tropics where native species are found.

The use of in vitro techniques would quite easily meet these demands. These techniques would allow for mass clonal propagation of superior varieties in a relatively short period of time. The elimination of seed and foliar borne pathogens would promote high yields of the crop.

Germplasm storage using micropropagated plants has advantages in that less space is required for preservation of plant material; maintenance cost of material is reduced and there is the reduced risk of loss of material through environmental factors, all of which are controlled in vitro.

This account outlines the preliminary investigations towards a general protocol for the regeneration of Yam Beans using in vitro techniques. The micropropagation on Pachyrhizus using single node culture was investigated with respect to several factors which included the effect of cytokinin on axillary bud burst; the effect of the addition of adenine to the culture medium and whether the position of the node along the stem influences the response of the explant to the medium. The potential for callus induction was examined with respect to the auxin used, explant age and physical environment on callus maintenance and development was investigated.

## MATERIALS AND METHODS

### Single Node Culture

Stem sections with axillary buds from the first to fifth nodal position from the apex of vigorously growing plants in the greenhouse, were surface sterilized in 15% sodium hypochlorite for 10 minutes, and rinsed three times in sterile distilled

water. Single nodes of each physiological age were placed on MS basal medium alone, and medium supplemented with one of several cytokinins, all at 1 mg/l concentration. The medium was solidified with 0.8% agar and pH regulated at 5.8 prior to autoclaving. The range of cytokinins used included Kin, BA, 2-ip, Zeatin and Adenine. Ten replicates per treatment were set up and incubated under 16 hr light period at 27$^{o}$C and 70% relative humidity. Four weeks after initiation of cultures, the explants were scored for axillary bud break or bud burst.

Callus Induction

Leaves from the first fully expanded leaf at the apex to the fifth leaf down the stem of the Yam Bean plant was sterilized with 10% sodium hypochlorite for 10 mins, and then rinsed three times in sterile distilled water. Discs of uniform size (14 mm diameter) were taken from leaves at each position and cultured on supplemented MS medium solidified with 0.8% agar, and pH adjusted to 5.8 prior to autoclaving. The basal medium was supplemented with several hormone combinations: 2,4-D alone, IAA plus Kin, NAA Plus Kin and 2,4-D plus Kin. All hormones were at 1 mg/l concentration. Ten replicates of each treatment were set up, half of which were incubated in complete darkness at 260C and the other half under a 16 hr light/8 hr dark cycle, at 27$^{o}$C and 68% relative humidity. The cultures were scored after 5 weeks for the presence of callus and the quantity produced.

Effect of Hormone, Carbon Source and Environment on Callus Development and Maintenance

Callus initially induced from leaf explants on solidified MS medium supplemented with 2,4-D and Kin (1 mg/l) was subcultured on MS with BA (1 mg/l). Two carbon energy sources, glucose and fructose were substituted for sucrose. Callus sub-cultured on both MS with glucose and fructose were incubated in the dark, and with 16 hr light period. Any significant differences in callus growth and development were noted four weeks after placing on medium with cytokinin.

Temporary squash preparations were made of callus incubated in both physical environments and were viewed under the light microscope to determine the nature of callus development.

Acetone extracts were made from callus that showed green coloration and the extracts were scanned with the spectro-photometer at wavelengths from 400-700 nm. The tracings obtained were compared to that of a standard prepared from primary leaf tissue.

## RESULTS AND DISCUSSION

### Single Node Culture

There was some contamination of the cultures by fungus, however, the surviving cultures were scored for bud burst. Table 1 shows the number of nodes which showed bud burst as a fraction of the surviving cultures.

The tabulated results indicate clearly that a cytokinin is required for bud burst to occur, since the hormone free medium did not stimulate the axillary buds to grow out. Bud burst is stimulated by the wide range of cytokinins used in culture. Adenine in this instance is acting as a cytokinin since it brought about the response normally attributed to cytokinin action, in inducing axillary buds to grow in culture. This growth regulatory property of adenine was first noted in 1939 by Bonner and Haagen-Smith and Bonner et al., 1939 (Sherrington and George, 1984), and was later demonstrated by Skoog and Tsui and Miller and Skoog (Sherrington and George, 1984).

Overall, the cytokinins BA, Kin and Zeatin are seen to be more effective in stimulating bud burst than the other two cytokinins used, BA showing a marginally better effect than Kin.

The nodal position of the buds, which is also indicative of the physiological age of the bud, did not have any significant effect on the ability to respond to the hormone stimulus present in the medium.

Multiple bud regeneration was apparent in some of the cultures after 5 weeks. The proportion of cultures showing multiple bud proliferation is expressed as a fraction of the number of cultures showing bud burst. The data is outlined in table 2.

An analysis of the data in Table 2 indicates that two cytokinins tested, 2-ip and Adenine are unable to stimulate multiple bud proliferation in Pachyrhizus. BA was thought to be the only cytokinin to induce this response, but on further investigation, Kin and Zeatin were also shown to be capable of stimulating this response. There is a position effect operating with respect to multiple bud regeneration capability. Buds at node 5 and to a lesser extent node 4 were incapable of this response to the hormone stimulus in the medium.

The effect of bud location on the success of micro-propagation through bud culture has been studied quite extensively and it has been found that explants at a young developmental stage are optimum for short regeneration (Hu and Wang, 1983). In Pachyrhizus bud location is critical only in the promotion of multiple bud regeneration, along with hormone type present in the medium.

Table 1. Effect of Hormone and Bud Age on Bud Burst of Pachyrhizus erosus.

| Nodal Position | Hormone BA | Kin | 2-ip | Zeatin | Adenine | None |
|---|---|---|---|---|---|---|
| 1 | 7/7 | 3/5 | 4/10 | 7/9 | 1/10 | 0/10 |
| 2 | 5/5 | 5/5 | 6/8 | 7/9 | 6/10 | 0/10 |
| 3 | 5/7 | 5/5 | 3/10 | 7/9 | 6/9 | 0/9 |
| 4 | 6/6 | 5/5 | 4/10 | 7/9 | 6/9 | 0/9 |
| 5 | 3/6 | 0/4 | 4/8 | 6/8 | 1/6 | 0/8 |

Table 2. Effect of Hormone and Bud Age on Capability for Multiple Bud Proliferation.

| Nodal Position | HORMONE BA | Kin | 2-ip | Zeatin | Adenine |
|---|---|---|---|---|---|
| 1 | 4/7 | 1/3 | 0 | 3/7 | 0 |
| 2 | 3/5 | 4/5 | 0 | 2/7 | 0 |
| 3 | 2/5 | 4/5 | 0 | 3/7 | 0 |
| 4 | 2/6 | 3/5 | 0 | 0 | 0 |
| 5 | 1/3 | 0 | 0 | 0 | 0 |

Callus Induction

The results indicate that of all the various auxins tested, NAA and 2,4-D in combination with Kin promote callus induction in Pachyrhizus. The results are tabulated in Table 3. Y represents presence of callus and N absence.

The presence of light is seen to be inhibitory to callus induction. The quantity of callus formed on medium with 2,4-D and Kin was much more than that on NAA and Kin, and was sustained more successfully over an extended period of time.

The quantity of callus produced from leaf explants of different ages was not accurately measured, but visually it was noted that with both hormone combinations promoting callus induction, the quantity of callus formed by explants taken from leaves 3-5 was much less than that formed from leaves 1 and 2.

In summary, callus induction in Pachyrhizus may be stimulated by either of two auxins 2,4-D and NAA in combination with the cytokinin, Kin, only in the absence of light. Leaf age is not a critical factor in callus induction, but it seems to have an effect on the rate of proliferation of callus.

Effect of Hormone, Carbon Source and Environment on Callus Maintenance and Development

The data in Table 4 indicates that fructose and glucose both support good callus growth, regardless of the environmental regime to which it has been subjected. There is however, a striking difference in the development of the callus which was exposed to a 16 hr light period when the carbon source was fructose.

The green color of the callus indicates the presence of chloroplasts, one of the most commonly found types of differentiation in callus cultures. The development of chloroplasts and hence chlorophyll pigment in tissue cultured cells is determined by the continued exposure to light and the carbon source used. Fructose has been shown to promote chlorophyll formation over glucose in combination with other factors, which may include the presence of cytokinin in the medium. The effect of hormone in chlorophyll formation has been reviewed by Sherrington and George (1984) who suggest that the presence of cytokinins tend to promote the formation of chlorophyll in callus or may even be essential for its formation in light.

The light stimulus is necessary for the differentiation of chloroplast and the formation of chlorophyll in cell culture. An account in Sherrington and George (1984) on chlorophyll formation in tissue culture, states that the chlorophyll precursor in the tissue culture system - protochlorophyllide - has an absorption

Table 3. The effect of Hormone and Light Regime on Callus Induction.

| Hormone combination | Presence of Callus | |
|---|---|---|
| | 16 hr Light | Dark |
| 2,4-D | N | N |
| 2,4-D + Kin | N | Y |
| NAA + Kin | N | Y |
| IAA + Kin | N | N |

Table 4. Effect of Environment and Carbon Source on Callus Development.

| Carbon Source | Physical Environment | Description of Callus Tissue |
|---|---|---|
| Fructose | 16 hour light | Large quantity of pale green friable callus |
| | Dark | Large quantity of friable brown callus |
| Glucose | 16 hr Light | Large quantity of friable brown callus |
| | Dark | Large quantity of friable brown callus |

aximum at 634 nm and is converted in red light to chlorophyll and also that in culture systems that may take the biosynthetic precursors of chlorophyll, the phytochrome system is probably responsible for converting protochlorophyllide into chlorophyll. The absorption of $P_r$ is 650-660 nm.

The squash preparations made, indicated the presence of three cell lines in the callus cultures. One cell line was made up of large, irregularly shaped and what optically appeared to be empty cells, the bulk of the callus being composed of these cells. The second cell line consisted of small, closely packed, isolated groups of cells that appeared to be actively dividing. There was, in addition to the two already mentioned, a group of cells that appeared green. These cells were present only in callus that had been exposed to light where the carbon energy source was fructose. The cells were fairly large with a prominent nucleus-like structure, around which were the green cell organelles, which gave the callus the green appearance.

All callus cultured on MS with BA indicated the presence of differentiated xylem tracheary elements, independent of the carbon source used and the environmental conditions to which they had been subjected. It appears, however, that the quantity of tracheids was more abundant in cultures incubated in the dark. These tracheids which were closely associated with the small actively dividing cells are most likely to be the ones undergoing meristematic activity, hence forming meristematic centres in the callus cultures. This may be further supported by the presence of tracheids in association with these areas, which may represent or be associated with an early stage in the development of shoot meristems (Sherrington and George 1984). The greater quantity of tracheids found in cultures in the dark indicates that there may be greater meristematic activity taking place in the dark. The type of carbon energy source does not appear to play any significant role in the differentiation of tracheary elements.

Cellular differentiation is an indication of meristematic activity in callus cultures, which is usually indicative of the potential for development of meristematic centres in the callus and subsequent shoot bud formation. All the callus cultures examined have signs that may suggest that there is the potential for plantlet regeneration from leaf callus of Pachyrhizus, but this has not yet been accomplished.

Analysis of the tracing made on the spectrophotometer from the callus extract (Appendix 1) and the standard leaf extract (Appendix 2), show both extracts with absorption peaks at wavelengths of approximately 435, 627 and 660 nm. This similarity confirms that the pigment in the callus is definitely chlorophyll. There is a difference, however, in the percentage absorbance at the wavelengths. The wavelengths at which the peaks occur suggest that more than one pigment system is involved, and the light may be intercepted by either the chlorophyll precursor or the phytochrome system.

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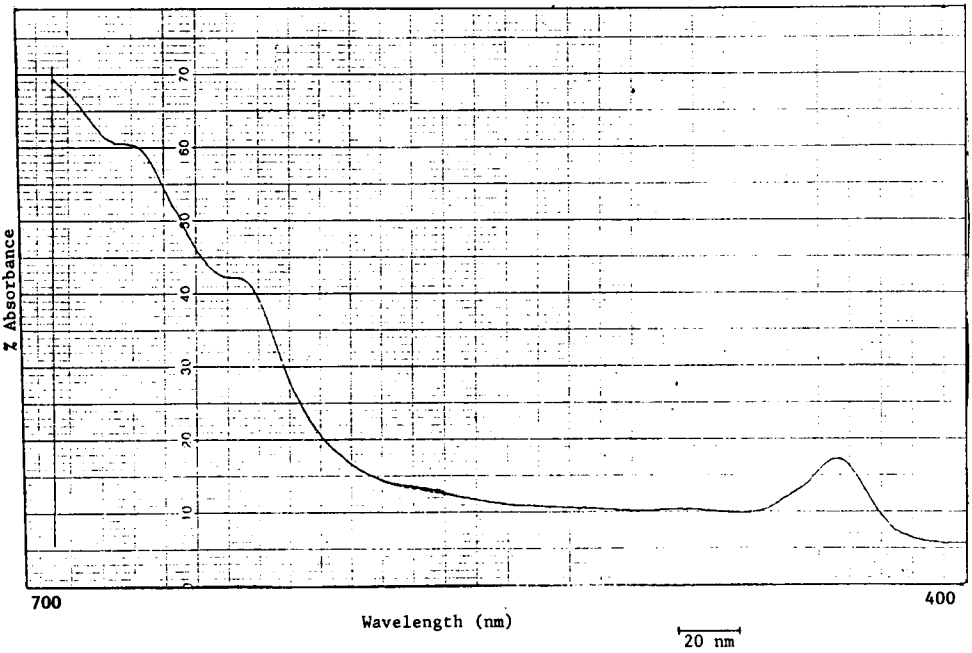
APPENDIX I

Spectrophotometer tracing of Acetone extract
of callus tissue of **Pachyrhizus**



APPENDIX II

Spectrophotometer tracing of Acetone extract
of primary leaf tissue of **Pachyrhizus**