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CALLUS INITIATION AND REGENERATION IN A MINOR TUBER CROP 'RIZGA' (*PLECTRANTHUS ESCULENTUS N. E. BR).*

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ABSTRACT

Calli were initiated from tuber cuttings, internodes and leaf disc explants of P.esculentus. From tuber cuttings, calli were formed when explants were cultured on MS basal salts supplemented with differing regimes of either 2,4–D and NAA separately or in combination with 0.5mgl^{-1} BAP. Initiation of callus was best when cultures were incubated on 2,4–D containing media. 2,4–D at a concentration of 1mgl^{-1} and culture condition of total darkness/25°C gave maximum calli fresh weight of 541.5 ± 43.9mg. In the case of internodes, maximum callus (82.3 ± 18.4mg) was obtained when explants were incubated on MS medium supplemented with 50gl^{-1} sucrose and 1mgl^{-1} 2,4–D. Leaf disc explants gave maximum calli fresh weights of 2,462.8 ± 279.7 and 1,688.8 ± 350.9 mg at sucrose concentration of 50 and 60gl^{-1}, respectively. Histological assessment of calli initiated from explants cultured on MS basal salts supplemented with 50gl^{-1} sucrose and 1mgl^{-1} 2,4–D and CP –a cytokinin, suggested caulogenesis and somatic embryogenesis might have occurred on CP and 2,4–D containing media, respectively.

Keywords: Callus, regeneration, tuber, *Plectranthus esculentus*

INTRODUCTION

Plant regeneration is the cornerstone of tissue culture methodology, without growth and differentiation studies, generation of genetically variable plants, commercial cloning for the purpose of rapid multiplication of desirable or difficult – to – propagate species, disease elimination through meristem culture, protoplast hybridisation and many growth regulators studies would not be possible (Dixon, 1985).

Many of the morphogenic responses are frequently associated with some degree of non – dedifferentiation which is expressed in the form of callus or callus – like tissues. Callus consists of an amorphous mass of loosely arranged thin – walled parenchyma cells arising from proliferating cells of explants cultured (Dodds and Roberts, 1990). It has also been interpreted as being highly meristematic pads of tissues which are not always completely dedifferentiatied into homogenous masses of uniform parenchyma – like cells. Various intermediate terms have been developed in the literature, for example, the term 'calloid' has been used by various workers to describe the compact nodular structures produced by tissue cultures of palms when explants are exposed to strong auxins such as 2,4-D (Blake and Hornung, 1995, Hornung, 1995).

Plant regeneration can be achieved through organogenesis (that is the formation and outgrowths of shoots – caulogenesis or roots – rhizogenesis) or via somatic embryogenesis (that is the formation of embryos from somatic tissues). These processes could either be indirectly from callus or directly from explants (Debergh, 1988). Organogenesis within callus cultures usually starts with the formation of clusters of meristematic cells (meristemoids) capable of responding to factors within the system to produce primordium (Torrey, 1966). Depending on the nature of the prevailing internal and external factors, the stimuli created can initiate either a root, a shoot or an embryoid. Localised meristematic activity, however, precedes organized development of either roots or shoots (Dodds and Roberts, 1990).

Until recently (Kyesmu and Mantell, 1998, 1999, 2001b, 2001c), there was no information in the literature about the tissue culture *of P. esculentus*. However, callus has been initiated from leaf disc explants of *P.esculentus* (Kyesmu and Mantel, 2001a).

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This paper, therefore, aims at developing both tissue and cell-based regeneration systems involving callus initiation and regeneration from tuber cuttings, internodes and leaf disc explants of *P. esculentus*. Success in these systems could lead to the evaluation of the feasibility of developing cell suspension culture system for *P. esculentus*. This system is an excellent starting materials for the isolation of protoplasts to be used in a wide range of applications such as hybrization through cell fusion, genetic manipulation – genetic engineering, etc (Hall, 1991).

MATERIALS AND METHODS

Plant materials

Freshly harvested tubers of *P. esculentus* were obtained in November 1992 and January 1993 from individual plants growing in the field plots belonging to local farmers in the Vom – Turu and Langtang districts. Vom – turu is situated about 20km south – west, while Langtang is about 240km south – east of Jos, the capital of Plateau state. Both sites are within latitudes 9° 00'N and 10° 30'N and longitudes 8° 30'E and 9° 30'E. Tubers were immersed for about 10s in 70% alcohol, then immersed and shaken in a hypochlorite solution with ca.0.01% detergent [Polyoxyethylene (20) – sorbitan monolaurate (Tween 20, BDH, UK)] added as surfactant for ca. 15 – 20min. The hypochlorite solution consisted of 10% (v/v) 'Brobat' bleach (Jeyes, UK) (containing 4.6% (v/v) sodium hypochlorite) made up with reverse osmosis water. After sterilisation, the tubers were cut into 5mm cube and these were then ready for innoculation onto sterile culture media in culture vessels.

In vitro donor plants for leaf discs and internodes were developed as described earlier by us (Kyesmu and Mantell, 1998). Leaf disc explants were obtained along midribs of leaves using heat – sterilised cork borer No. 3 (6mm diameter). Internodes of 5 – 10mm in length were obtained from the same *in vitro* plants from which leaf discs were excised. The internodes were placed horizontally on the surface of the media while the adaxial surfaces of leaf discs were placed in contact with the surfaces of the media as reported by us (Kyesmu and Mantell, 2001a).

Treatments

i) Tuber cuttings were cultured in flat – bottomed, rimless boro – silicate glass tube (25 x 75mm) containing 10ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30gl^{-1} sucrose and varying concentrations (0, 0.5, 1, 3 and 5mgl^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D) and α–naphthalene acetic acid (NAA) separately and also in combination with 0.5mgl^{-1} 6–benzylaminopurine (BAP). Cultures were incubated under either total darkness or 16h photoperiod at 25°C for 35 days.

ii) The inferences drawn from the experiment in i) above, were then applied to internodes and leaf discs to determine the effects of different sucrose levels as well as 2,4-D concentrations on primary callus initiation. In this investigation, the effects of 2,4-D when used at 0, 1, 2, 3, 4, 5 and 10mgl^{-1} in combination with 30, 50 and 60gl^{-1} sucrose on callus induction from leaf and internoda explants were evaluated.

iii) Calli initiated from the resulting experiment in ii) above were repeatedly subcultured and subsequently assessed to determine callus differentiation. In addition calli were also obtained from leaf disc cultured on MS basal salts supplemented with N-(2-chloro-4-Pyridyl)-N'-phenylurea (CP – a cytokinin). Leaf disc excised from the middle sectors of leaves of 42-day old *in vitro* shoot cultures were cultured on MS basal salts supplemented with 50gl^{-1} sucrose and either 2,4-D or CP at 1mgl^{-1}. Cultures were incubated under total darkness at 25°C. Histological assessment of nodular calli were carried out after 35days using the methodology of Johansen 1940 as described by Kyesmu and Mantell (2001b).

Statistical analyses

Statistical analyses were performed using the Agstats statistical programme (developed by A. Clewer of Wye College Computer Services Unit, University of London, UK). Graphs were drawn using Microsoft Excel computer programme (bars on graphs denote standard error). Generally twenty culture tubes were used for every experimental unit. Qualitative assessment was employed in the case of calli texture while fresh weights were

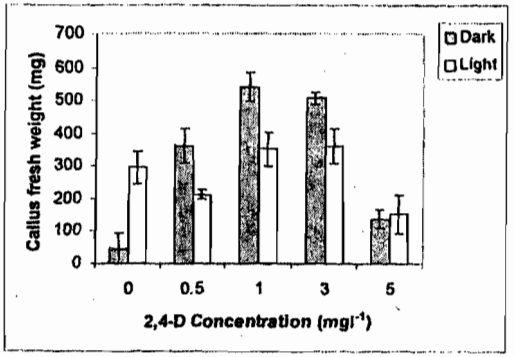

Figure 1. Effects of 2,4-D concentration (mgl^{-1}) on callus initiation from tuber cuttings of *P.esculentus* after 35 days at 25°C

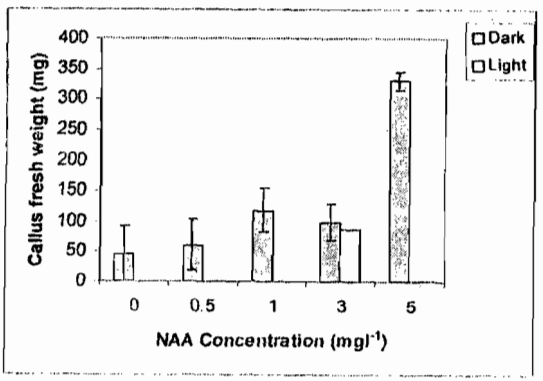

Figure 2. Effects of NAA concentration (mgl^{-1}) on callus initiation from tuber cuttings of *P.esculentus* after 35days incubation at 25°C

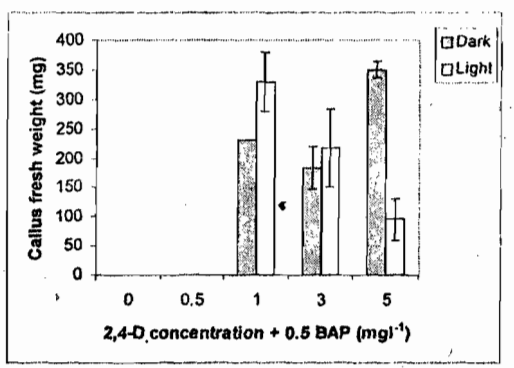

Figure 3. Effects of 2,4-D concentration in combination with 0.5mgl^{-1} BAP on callus initiation from tuber cuttings of *P.esculentus* after 35days incubation at 25°C.

measured using a Sartorious top – pan electronic balance.

RESULTS

Tuber cuttings

Figures 1 and 2 illustrate the effects of 2,4-D and NAA on primary callus induction from tuber cuttings of *P. esculentus*. Figures 3 and 4 further shows the auxins-cytokinin interactions. In 2,4-D media, callus initiation was maximum at 1mgl^{-1} (541.2 ± 0.04mg, callus fresh weight) when the cultures were incubated under total darkness at 25°C (Figure 1). When in combination with 0.5mgl^{-1} BAP, callus induction was reduced (230.7 ± 0.0mg). In the presence of BAP, maximum initiation was obtained when 2,4-D was at a concentration of 5mgl^{-1} (349.1 ± 0.13mg) and also when cultures were incubated under total darkness at 25°C (Figure 2).

In NAA containing media, callus initiation was enhanced only when NAA was in combination with 0.5mgl^{-1} BAP. Maximum initiation was obtained at a concentration of 3.0mgl^{-1} (510.2 ± 0.11) (Figure 4). Without BAP, callus induction was maximum at a concentration of 5mgl^{-1} (349.1 ± 0.13mg) (Figure 3).

Calli obtained from cultures incubated on 2,4-D containing media and incubated under total darkness were mostly yellowish – brown. Under 16h photoperiod, the calli were compact and greenish – white. Those obtained form NAA

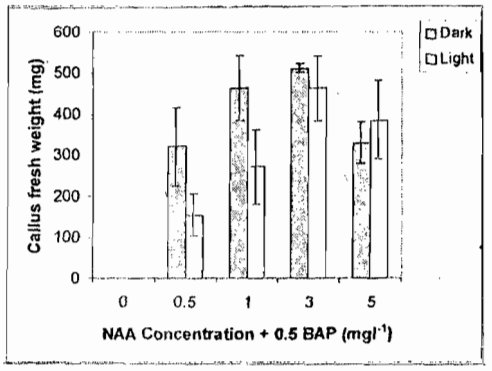

Figure 4. Effects of NAA concentration in combination with 0.5mgl^{-1} BAP on callus initiation from tuber cuttings of *P esculentus* after 35days incubation at 25°C.

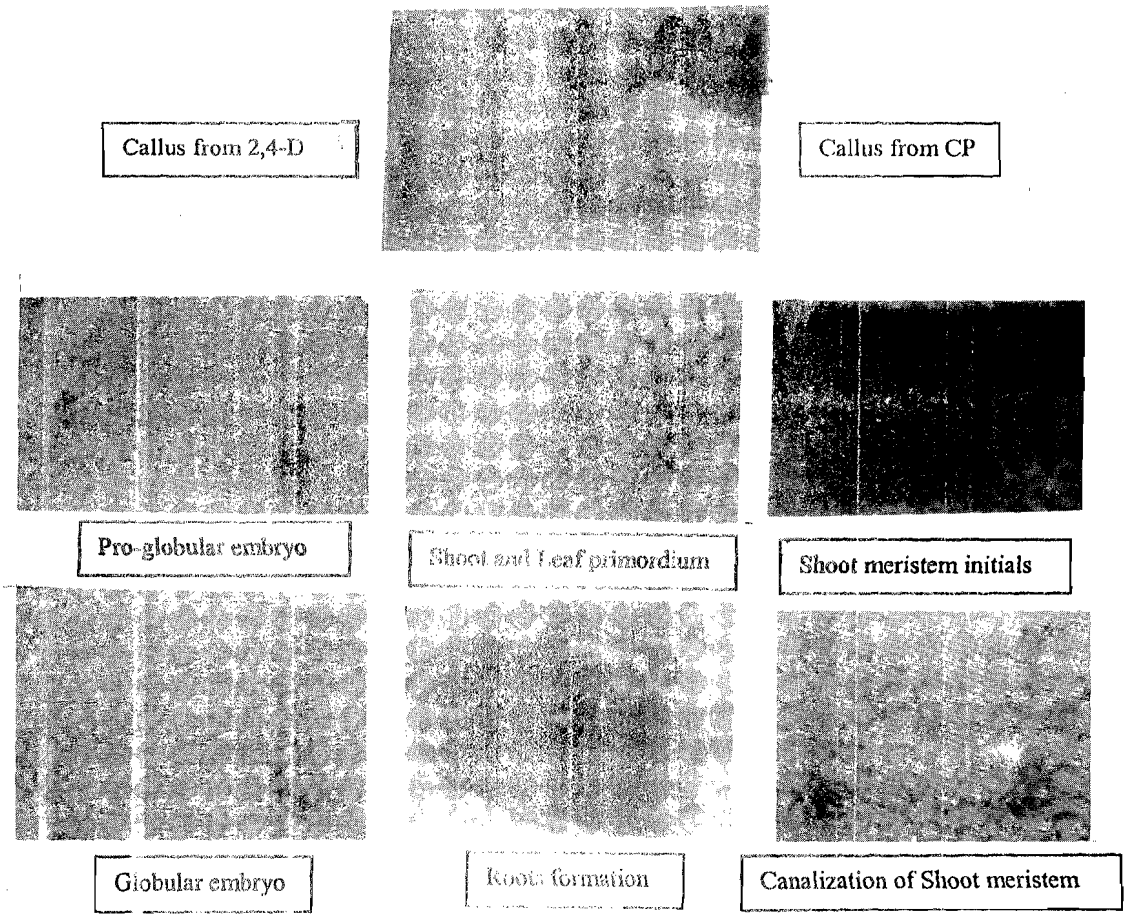

Plate 1 Sections through callus produced on a 2,4-D and CP containing media after 28 – 30days incubation. A series of profiles showing stages in the formation of pro – globular, globular embryo and root formation – root cap with densely packed meristematic cells (2,4-D callus). Another series of profiles of a single morphogenic site demonstrating the presence of developing shoot meristem via organogenesis on callus from CP.

containing media were slightly compact, watery, globular with roots produced. With the addition of BAP in the media, compact fine greenish white calli were produced.

Internodes and leaf disc

Results showed that of all 2,4-D levels tested, 1mgl^{-1} of this auxin supported callus induction across all levels of sucrose tested. Leaf discs produced calli across all 2,4-D levels tested when sucrose levels were either 30 or 50gl^{-1}. At 60gl^{-1}, calli were produced only at 2,4-D concentration of 1, 2, and 3mgl^{-1}. By contrast, internodes were only able to induce calli at either 0 or 1mgl^{-1}. This indicated that 2,4-D levels above 1mgl^{-1} inhibited callus response in internodes. In the case of leaf disc explants, inhibition was only observed at concentrations above 3mgl^{-1} in media with 60gl^{-1} sucrose (Table 1). Relative fresh weights of calli were greater in cultures of leaf disc

Table 1. Effects of MS basal salts supplemented with sucrose and 2,4-D on callus initiation from internodes and leaf disc explants of *P.esculentus.*

Sucrose (gl^{-1})	2,4-D Concentration (mgl^{-1})	Callus $_{Internode}$ (mg)	Callus $_{Leaf\ Discs}$ (mg)
30	0	19.33 ± 0.50	19.33 ± 0.50
	1	75.22 ± 16.80	361.4 ± 37.70
	2	0	134.4 ± 40.50
	3	0	133.7 ± 34.50
	4	0	82.3 ± 16.50
	5	0	71.7 ± 16.50
	10	0	82.3 ± 16.50
50	0	0	19.7 ± 1.1
	1	82.3 ± 18.4	**2462.8 ± 279.7**
	2	0	366 ± 39.8
	3	0	354.5 ± 39.8
	4	0	386 ± 51
	5	0	343 ± 39.8
	10	0	196 ± 107.3
60	0	0	0
	1	18.7 ± 0.5	**1688.8 ± 350.9**
	2	0	343 ± 39.8
	3	0	86.6 ± 14.3
	4	0	0
	5	0	0
	10	0	0

NB: Explants were incubated under total darkness at 25°C for 35days.

than those in internodes. The maximum amount of calli were produced when both types of explants were cultured on media containing both $1mgl^{-1}$ 2,4-D and $50gl^{-1}$ sucrose.

The qualities (that is appearances and textures) of calli appeared to be different depending on the explant type used and the relative levels of 2,4-D and sucrose present in the culture media. Calli produced from leaf discs were in general nodular and loosely textured, while those produced from the proximal and distal ends of internodes were less nodular and more compact in appearance and texture.

Histological assessment

When leaf disc explants were cultured on MS basal salts supplemented with either 2,4-D or CP at $1mgl^{-1}$ and $50gl^{-1}$ sucrose, and incubated under total darkness at 25°C, calli produced were substantially different with respect to their colours and textures. Calli produced from leaf disc cultured on 2,4-D media were yellowish – brown, nodular and granular in texture, while those calli from CP media were creamy white, soft and fine textured.

Calli induced on CP media and then subcultured on plain MS and incubated under 16h photoperiod or total darkness conditions produced shoots, whilst those calli produced on 2,4-D media when subcultured on plain MS and incubated at either 16h photoperiod or total darkness at 25°C produced roots. However, histological assessment of calli sampled from both 2,4-D and CP culture

treatments indicated that both organogenesis and somatic embryogenesis might have taken place in *P.esculentus* explants. To establish this, histological assessment of representative samples of cultured explants and resultant examination of paraffin-embedded sections of the samples are presented in Plate 1. Evidence obtained indicated that shoot formation was common in leaf explants cultured on media supplemented with CP, while somatic embryogenesis could have possibly occurred on calli of leaf explants cultured on media supplemented with 2,4-D. However, structures that had developed into pro-globular were observed, the globular structures would later develop into pro-embryo and sunsequently embryos. But because the calli obtained in this study were left on MS media containing 2,4-D for up to 20days and above before being transferred to plain MS, the structures began to develop root primordia while shoot primordia appeared to be suppressed. After 28days, root initials and later roots were commonly observed on most cultures (Plate 1.)

DISCUSSIONS AND CONCLUSION

The results of the current investigation indicated that greatest callusing responses from tuber cuttings were obtained when explants were cultured on MS supplemented with $1mgl^{-1}$ and when cultures were incubated under total darkness at 25°C, the callus obtained were mostly compact and yellowish brown. In the case of internodes and leaf disc explants, maximum callus initiation was obtained when explants were cultured on MS supplemented with $1mgl^{-1}$ 2,4-D and $50gl^{-1}$ sucrose. Gleddie *et al.* (1982) while working on leaf explants of *Solanum melongena* observed callus induction when explants were cultured on MS medium supplemented with 0.1 – $10mgl^{-1}$ of either NAA, 2,4-D or 2,4,5-T. Considerable callus initiation was observed after 20days incubation. Callus initiation was favoured under total darkness at 25 or 30°C. Auxins are generally required to be incorporated into culture medium for the induction of callus from explants (George, 1993 and Dodds and Roberts, 1990). Irvine *et al.* (1983) having tested 79 potential regulants for the ability to initiate callus from immature sugar cane leaf tissues observed that the regulant that most frequently initiate callus was 2,4 – D and it is generally used at levels between 1 and $3mgl^{-1}$. This same auxin was observed to induced callus from explants of *P.esculentus*. Whereas auxins have been known for their callus and roots induction process, cytokinins have been reported to be very effective in promoting either direct or induced shoot initiation. However, little concentration of it in combination with slightly higher concentration of auxins has been reported to enhanced callus initiation. This depends on the type of auxin used (Dodds and Roberts 1990). From the current study on *P.esculentus*, while the additon of $0.5mgl^{-1}$ BAP to differing concentrations of 2,4 – D does not seem to enhance callus induction (Figure 3), in the case of NAA appreciable callus induction was observed (Figure 4). Rossi – hassani and Zryd (1995), however, observed rapidly growing calli from internodes, leaf and sepal-derived explants of *Portulaca grandiflora* when these were cultured on MS medium supplemented with a combination of either 3.0μM 2,4-D and 5.0μM Kinetin (KIN) or 5.0μMNAA and 10μMKIN. Calli were visible after two weeks along the explants' cut edges. The growth regulators were reported to have affected calli texture, for example, a combination of 3.0μM 2,4-D and 5.0μMKIN gave a white friable ccalli, while a combination of 5.0μMNAA and 10.0μMKIN favoured the formation of green compact callus.

The effects of CP in the current study was directly linked with shoot morphogenesis.CP has generally been discovered to be active in promoting callus growth and morphogenesis in tobacco and several other kinds of plants (Okamoto et al., 1978; Takahashi et al., 1978; Kamada and Harada, 1979; Ohyama and Oka, 1982).

In the light of the above discussions and investigations carried out on callus initiation and regeneration of *P.esculentus*, it would appear that leaf disc explants were preferable to internode and tuber cuttings from the point of view of producing appropriate types of calli for liquid cell suspension culture work. Calli produced from leaf disc explants were predominantly loose textured calli, which could be easily dispersed in a liquid medium. Success in this system indicates that preliminary evaluation of the feasibility of developing cell suspension and protoplast culture for *P.esculentus* could be carried out. However with further extension of culture incubation period from 28 – 30 days, the callus so produced underwent *de novo* regeneration. Callus produced from 2,4 – D

subsequently produced roots with histological assessment suggesting possible somatic embryogenesis. While callus from CP produced shoots (Plate 1). These systems could be evaluated for *in vitro* selection against appropriate stresses such as drought, infection etc.

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