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ANALYSIS OF PROTEIN AND PEROXIDASE FROM EMBRYOGENIC AND NON-EMBRYOGENIC CULTURES OF *CITRUS RETICULATA* L.

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Cotyledons excised from 18 days old seedlings were used as explant source for callus induction in *Citrus reticulata* L. c.v. *Kinnow mandarin*. Various media combination were tested. The modified Murashige and Skoog media containing 2,4-dicholorophenoxyacetic acid (2,4-D) 1.0 mg/l, benzylaminopurine (BAP) 0.5 mg/l and naphthaleneacetic acid (NAA) 0.5 mg/l proved to be optimum for callus formation. Non-embryogenic calli were noted during first three passages and embryogenic calli started initiating from 4th passage and continued even after prolonged subculturing. Biochemical analysis revealed that quantitative differences existed in proteins both in embryogenic (E) calli and total non-embryogenic (NE) calli. Total protein quanity (µg/g culture) drastically decreased till the 3rd passage and progressively increased later on. The embryogenic culture also showed higher level of peroxidases activity.

Key words: *Citrus reticulata* L., Cotyledon, Callus, Non-embryogenic, Protein, Peroxidase.

Introduction

Biochemical studies of embryogenesis has been very limited, but there are certain markers to distinguish embryogenic calli (E. calli) from non-embryogenic calli (NE calli). These markers include peroxidases, polyphenol oxidases and other proteins [1]. Chen and Luther reported that there were some qualitative and quantitative differences between the proteins present in E. calli and NE calli and in embryos of rice [2]. Several biochemical changes have also been observed during somatic embryogenesis in carrot cultures. These changes include rate of RNA and protein synthesis which are greater in E. calli than NE calli [3,4]. In addition to proteins, isozymes can be employed as effective markers on differentiation. Most of the studies have been carried out only on a few isozyme systems, most frequently the peroxidases system. Recently, the possibility of commercial production of peroxidases by plant cell tissue culture techniques has been discussed, in the case of horse-radish cells [5] and from radish *Raphanus sativus* cells [6]. Extra cellular peroxidase has been reported in suspension cultures of carrot [7], peanut [8], potatoes [9] spanish [10], cotton [11], radish [6], pepper [12] and also in tobacco callus [13]. It has also been found in suspension cultures of cowpea where different growth regulator concentrations induced different peroxidases activity in callus [14]. The specific objective of the study was to determine any difference in the total protein and peroxidase present in E and NE calli derived from cotyledons of *in vitro* seedling of *Citrus reticulata* L. cv. *Kinnow mandarin*.

Materials and Methods

Preparation of plant material. The seed were excised from the mature fruit of plants growing at the citrus plantation of National Agricultural Research Centre, Islamabad. They were surface sterilized for 30 sec. by 70% ethanol followed by a 15 min. asepsis in 2.65% sodium hypochlorite, to which few drops of Tween 20 were added. Traces of detergents were removed by three times washing with autoclaved distilled water. Seeds were germinated by culturing on Murashige and Skoog (MS) basal medium [15] and incubated at 24°.

Callus culture. Cotyledons were excised from 18-days-old seedlings and cultured on media containing 10 ml of modified MS callus induction medium (CIM). For embryogenic callus production 2,4-D, NAA and BAP were added. The cultures were incubated with 16 hrs photoperiod at 24° + 2°. Subsequent subculturing of both embryogeic and non-embryogenic calli was carried out after every 4 weeks.

Peroxidase assay. Peroxidase activity was estimated by using the guaicol H_2O_2 method of David and Murray [16]. One gram of callus was crushed in an ice chilled mortar together with 5 ml cold 0.1 M phosphate buffer. The slurry was filtered through 4 layers of cheese cloth and the filtrate was centrifuged at 14,000 rpm for 20 mins at 4°. The supernatant was decanted and was immediately used for the enzyme assay.

Protein estimation. Total protein was determined by using biuret method of Roenson and Jhonstone [17]. One gram of callus was crushed in an ice chilled pestle mortar in the presence of 0.1 m dipottasium hydrogen phosphate buffer. Slurry so obtained was centrifuged at 10,000 rpm for 10 mins at 4° and the supernatant was used for protein estimation.

Results and Discussion

Callus culture. Cotyledons excised from *in vitro* seedlings were cultured on modified MS medium supplemented with different concentrations of growth hormones. The best

response for callogenesis was observed on media containing 1.0 mg/1 2, 4-D, 0.5 mg/1 NAA and 0. mg/1 BAP. Callus initiation was observed from the greenish part of the cotyledon. The primary calli formed after 4 weeks were mostly non-embryogenic and were maintained on the callus inducing medium (CIM) from which NAA was omitted. Most of the calli produced were non-embryogenic upto three passages (Fig. 1). At the 4th passage, there was a gradual switch of the cultures towards embryogenesis which was maintained 7th subculturing (Fig. 2). These results show that citrus, like many other species, is capable of producing 2 types of calli i.e. embryogenic (E) and non-embryogenic (NE). Embryogenic cultures are defined as having the capacity to form somatic embryos and subsequently, regenerate into plantlets when placed on the appropriate medium. Non-embryogenic calli, on the other hand, grows in an unorganized manner and may occasionally give rise to shoot or roots by organogenesis [18].

Fig. 1. Non-embryogenic callus formed from *in vitro* derived cotyledons of *Citrus reticulata* L/cv. Blanco.

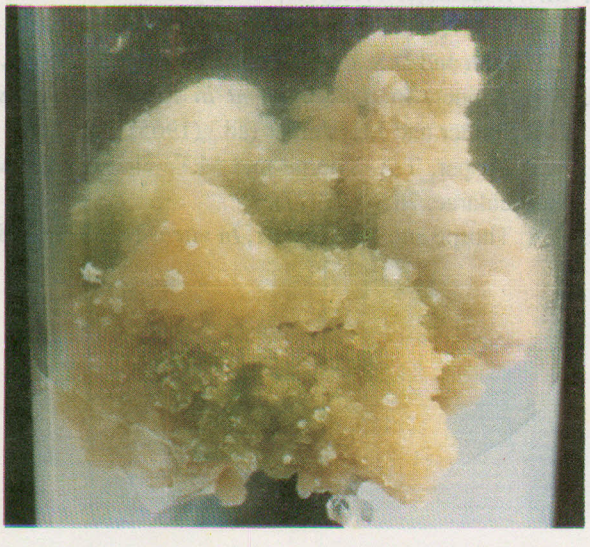

Fig. 2. Embryogenic callus formed from *in vitro* derived cotyledons of *Citrus reticulata* L/cv. Blanco.

Biochemical analysis. The quantitative analysis of protein (µg/g of culture) and the peroxidase estimation was carried out on both embryogenic and non-embryogenic calli upto 7th passage to confirm that there were biochemical differences between the 2 callus types. The amount of protein (µg/g fresh weight of culture) was almost the same in zero culture and control (cotyledons). This could be explained by assuming that dedifferentiation does not originate in zero culture. Drastic decreases in protein contents were observed at the first subculture. The amount of protein decreased progressively upto 3 subcultures. Protein quantity increased from the 4th subculture and continued increasing till the 7th passage (Fig. 3 a,b). This increase is directly related to embryogenic nature of the culture. Chen and Luthe [19] reported similar results in rice. Amount of protein in E. calli was 1.6 fold greater than NE calli. This was probably due to the greater water content of NE callus [19].

The peroxidase activity of *in vitro* derived calli showed that its quantity decline as the degeneration or dedifferentia-

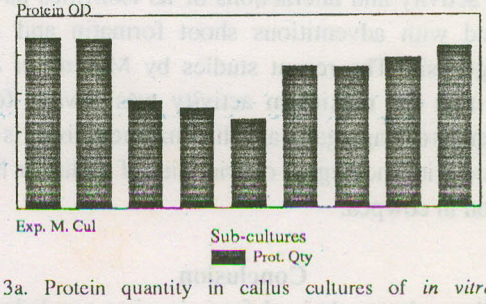

Fig. 3a. Protein quantity in callus cultures of *in vitro* derived cotyledons.

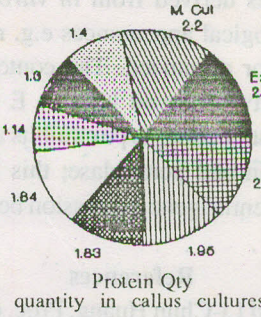

Fig. 3b. Protein quantity in callus cultures of *in vitro* derived cotyledons.

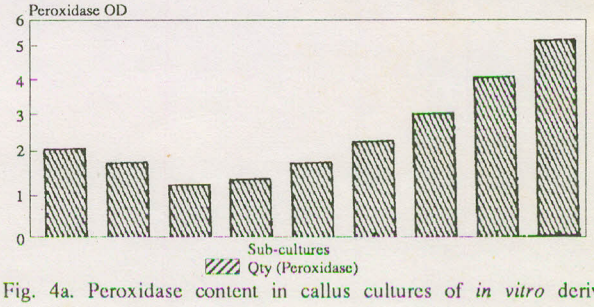

Fig. 4a. Peroxidase content in callus cultures of *in vitro* derived cotyledons.

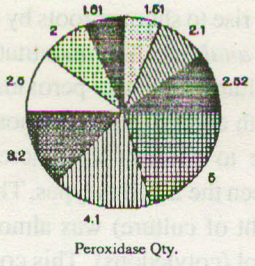

Fig. 4b. Peroxidase content in callus cultures of *in vitro* derived cotyledons.

tion or callus formation begins. According to Bonner [20] and Gaspar [21] the changes in isozyme patterns are the cause rather than the result of de-differentiation. Our study highlighted that the activity of peroxidases, simultaneously increases with the differentiation except at 4th passage due to necrosis (Fig. 4a,b)

Similar results were obtained following the investigations of Thorp and Gaspar [22] who reported that an increase in peroxidase activity was correlated with the adventitious shoot formation and somatic embryogenesis. Increase in peroxidase activity and alteractions of its isozymes have been correlated with adventitious shoot formatin and somatic embryogenesis. The recent studies by Moreno *et al.* [14] showed that the maximum activity was always found in homogenous cell agregates and this enzyme activity is directly correlated with the degree of friability of callus or NE calli formation in cowpea.

Conclusion

Cellus cultures derived from *in vitro* cotyledons have different morphological appearances e.g. rough or unorganized and smooth or organized. The content of protein and peroxidases activity observed both in E and NE calli has revealed that the latter callus type exhibits an increased quantity of both protein and peroxidase; this increase could be attributed to differential gene expression between the 2 callus types.

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