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COMPARISON OF TRYPSIN PROTEASE INHIBITOR SYNTHESIS PRODUCED FROM *IN VITRO* CULTURE AND NATURAL *COCCINIA INDICA* W&A

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Plants have evolved a defense mechanism that interferes with the insect's digestive system by expressing a number of different protease inhibitors (PIs), which are either constitutively present or strongly up-regulated in response to wound damage. In herbivorous insects they act by inhibiting protein digesting enzymes in the guts of insect larvae or adults, resulting in amino acid deficiencies that lead to serious developmental delay, mortality or reduced fecundity. Their physiological roles in the plants seem to be the control of proteinases during seed dormancy and protection against the proteinases of many parasites and insects. Protease inhibitors have been shown to have strong anticarcinogenic activity in *in vivo* and *in vitro* cancer model systems. Relatively little is known about the precise mechanism(s) by which these compounds exert their suppressive effects. *Coccinia indica* W&A (Cucurbitaceae) is a wild tropical plant and the fruits are considered as vegetable. In the present paper we describe the callus cultures with the aim to overproduce trypsin protease inhibitor. Murashige and Skoog (MS) medium with various hormone combinations were found effective in initiating callus tissue. NAA/BA combinations were found more efficient among different hormonal regimes, such as NAA/BA, IAA/BA, IBA/BA and 2, 4-D kinetin. Callus tissue developed successfully in 12 hr/12 hr light-dark regime as well as in the dark. Trypsin protease inhibitor activity is quantified by inhibiting the activity of trypsin. Biochemical analysis of the callus tissue showed two fold increase in the amount trypsin protease inhibitor as compared to that from fresh plant material. Calli of leaf generally showed higher protease inhibitor activity. Protease inhibitor activity was highest in leaf calli derived from IBA/BA combinations. This reveals the usefulness of tissue culture as a system to trigger the over expression of trypsin protease inhibitior gene in *C. indica*. Strategies such as variations in media components, additives, use of elicitors, etc are expected to further increase the trypsin protease inhibitor yield in tissue culture several fold. *In vitro* culture of this species might offer an alternative method for production of these important pharmaceuticals, which would reduce the collection pressure on this plant.

Keywords: Anticarcinogenic activity; BA; Callus; 2, 4- D; IAA; IBA; Kinetin; NAA; TPi.

Introduction

Plants are a valuable source of a vast array of chemical compounds; they synthesize and accumulate extractable organic substances in quantities sufficient to be economically useful as raw materials for various commercial applications. Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to depletion of habitat and ruthless collection, medicinal plants are on the verge of extinction. Hence, the conservation of these valuable genotypes is imperative. Plant tissue culture technology holds great promise for micropropagation, conservation, and enhancement of the natural levels of valuable primary / secondary plant products and to meet pharmaceutical demands and reduce the in situ harvesting of natural forest resources. For mass propagation of medicinal plant species in which conventional methods possess limitations, *in vitro* multiplication provides the way out. There are sufficient reports available about protocols on *in vitro* micropropagation of many medicinal species[1,2].

The protease inhibitors are small proteins which inhibit the proteolytic activities of mammalian pancreatic digestive proteinases. The majority of proteinase inhibitors studied in the plant kingdom originate from three main families, namely Leguminosae, Solanaceae, and Gramineae. Plant PIs are well known to play a potent defensive role against predators and pathogens. Diverse endogenous functions for these proteins

have already been proposed, ranging from regulators of endogenous proteinases to storage proteins, but evidence for many of these roles is partial or confined to isolated examples[3]. In addition, many plant PIs have been shown to act as defensive compounds against insects by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has accumulated consistently[4]. The role and mechanism of action for most of these inhibitors have been, or are being, studied in detail, and their respective genes have been isolated. These genes have been used for the construction of transgenic crop plants to be incorporated in integrated pest management programs[4,5]. Given the number of pesticidal proteins involved in host plant defense, effective pest control by this strategy will presumably result from the co-expression of numerous determinants, each of which could be custom engineered by directed molecular evolution to maximize its effectiveness against specific pests.

Coccinia indica belongs to the family cucurbitaceae commonly found in India, Pakistan and Srilanka. It is a climber and trailer. The fruit of *Coccinia* is used as a vegetable, when tender and is also eaten fresh. When ripened the fruit turns into a bright scarlet colour. A literature survey indicated that the *in vitro* protocol for stem/ leaf culture of this climber was not yet standardized. In view of its medicinal importance, and the lack of tissue culture reports, the present study reports the prime protocol for regeneration from stem/ leaf explants of *Coccinia indica* W & A. and to conduct an *in vitro* analyses of the proteinase inhibitors under varying experimental conditions.

Material and Methods

Plant material: Coccinia indica growing wild was used to initiate callus cultures. Young plants of *C. indica* were brought to the laboratory under ice. The plants were pre-sterilized in teepol for 5 min and washed thoroughly in running tap water. Then the juvenile stem and leaf were disinfected with 70% ethanol (v/v) for 4 min followed by 0.1% $HgCl_2$ (6 min) and rinsed several times with sterilized distilled water. The internode and leaf were excised and cut into pieces of 0.5 cm. Tissue cultures of *C. indica* were initiated in MS medium[6]. For initiating calluses the auxins, Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA) 2, 4-Dichloro phenoxy acetic acid were used alone and in combinations with the cytokinin 6-Benzyl adenine (BA). However, for 2, 4-D combinations kinetin was used as the cytokinin. A range of concentrations from 0.1 to 5mg/l of each hormone was used.

Isolation and assay of trypsin protease inhibitor: Trypsin protease inhibitor was isolated and assayed from plant material and calluses developed from different hormonal combinations as per the methodology of Kakade *et al.* [7].

Result and Discussion

Callus growth: Leaf and internodal explants cultured on MS basal medium facilitated initiation of callus. The combinations with higher auxin and lower cytokinins are able induce calli effectively. Different hormones and their combinations were tried to find their effect on the *in vitro* production of proteinase inhibitors. The calluses induced by different hormone combinations varied in their morphology. MS media supplemented with all concentrations of auxin or combination of auxin with cytokinin tried in the present study facilitated callus initiation. IAA was more effective than BA, when used singly. Smith *et al*[8]developed calluses successfully from cotton and reported the efficiency of IAA in inducing callus. 2, 4 D / kinetin and NAA/ BA combinations were successful in inducing highest calluses (Figs. 1-4). IAA/BA combinations produced greenish white calluses with root formation. Generally calluses with root formation will not show further regeneration. However, root formation is an organogenetic process can influence the primary or secondary metabolism in the callus. The callus induced on green leaf explants grew slowly in the initial phase in the form of a lumpy mass but later becomes compact.

IBA was able to induce callus with root formation at medium with higher concentrations. IBA is generally the auxin of preference used to induce roots during regeneration[9]. However, IBA induced rooted calluses not be used for further regeneration. Generally IBA induced calluses were smaller than the calluses induced by other auxins used in the present study. Increase in the concentrations of BA from 0.1 to 0.5 mg/l did not affect callus formation.

NAA alone at lower concentration of 0.1 mg/l was able to induce callus. However, BA along with NAA proved most successful in inducing and supporting callus growth in most of the combinations tried. Maximum callus growth in the present investigation was observed in cultures with 1 mg/l NAA and 0.1 mg/l BA. Generally leaf explants showed better callus induction response than internodal explants. The calluses were yellowish-white, friable and devoid of roots. Such calluses were reported to be useful to initiate cell suspensions.

Trypsin protease inhibitor assay (*TPi*) : Trypsin protease inhibitory activity is quantified indirectly by inhibiting the catalytic activity of trypsin by the methodology of Kakade *et al*[7]. Undifferentiated and partially differentiated *Coccinia* callus were tested for the presence of Trypsin protease inhibitors. The result of the analysis was shown in Figs. 5-8. Trypsin protease inhibitory activity of callus tissue showed a two fold increase compared to that from *in vivo* analysis.

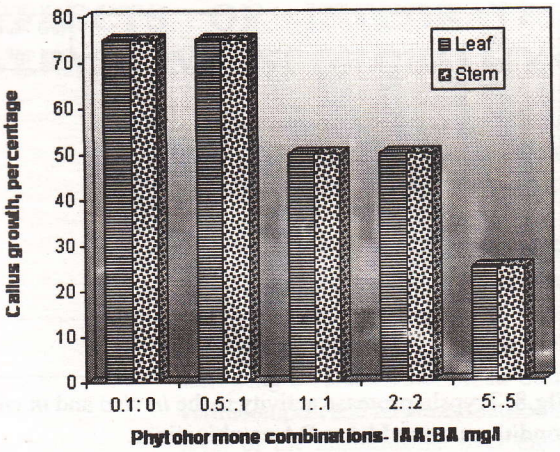

Fig.1. Callus growth from leaf and stem explants in MS medium with different IAA/BA combinations.

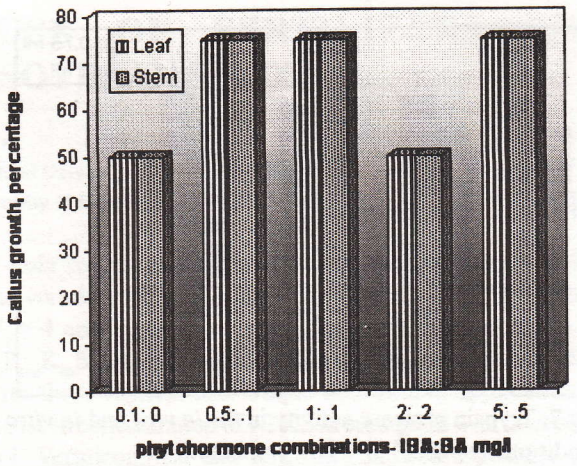

Fig.2. Callus growth from leaf and stem explants in MS medium with different IBA/BA combinations.

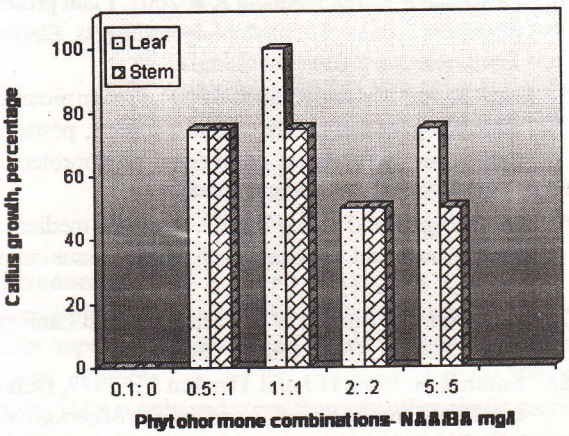

Fig. 3. Callus growth from leaf and stem explants of *C. indica* in MS medium with different NAA/BA combinations.

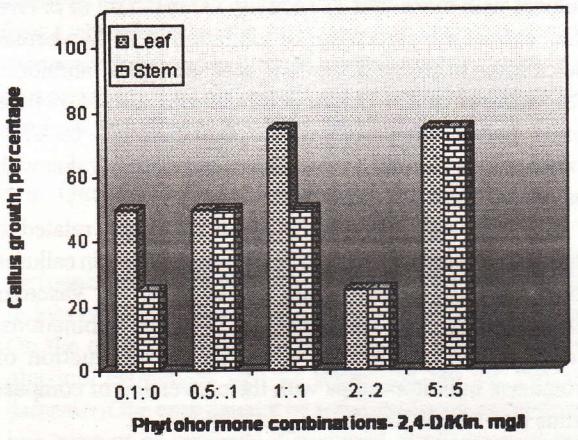

Fig.4. Callus growth from leaf and stem explants of *C. indica* in MS medium with different 2,4-D/Kin. combinations.

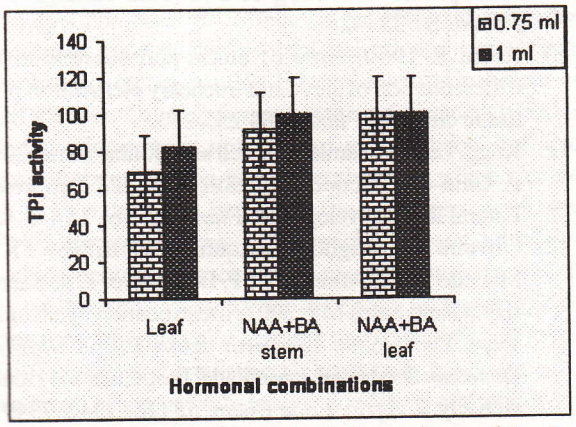

Fig.5. Trypsin protease activity in the *in vivo* and *in vitro* conditions using NAA + BA combinations.

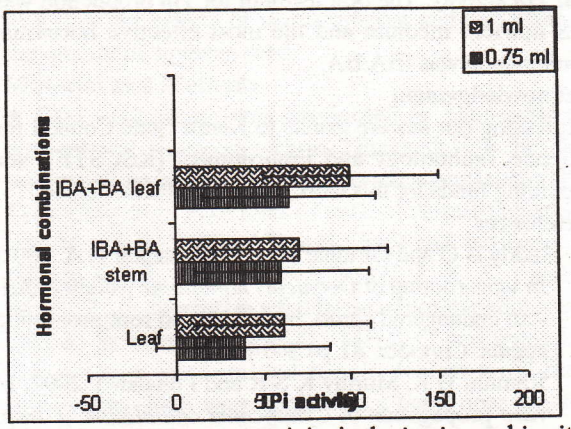

Fig.6. Trypsin protease activity in the *in vivo* and *in vitro* conditions using IBA + BA combinations.

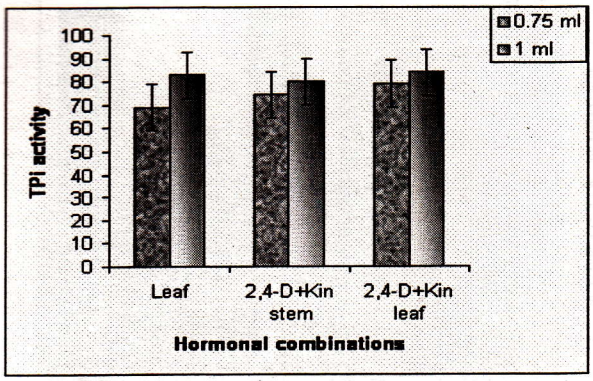

Fig.7. Trypsin protease activity in the *in vivo* and *in vitro* conditions using 2,4-D + Kin combinations.

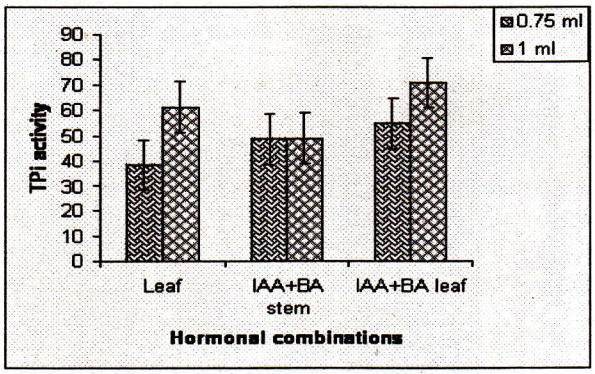

Fig.8. Trypsin protease activity in the *in vivo* and *in vitro* conditions using IAA + BA combinations.

In NAA/BA combinations the TPi showed 100% inhibitions in 0.75 ml extract when compared with the *in vivo* plant (60%). IAA/BA combinations produced the calluses with maximum TPi activity i.e. in 0.5 ml of *in vivo* plant extract the percentage of inhibition is 50 % whereas the calluses of the combinations produce 100% inhibition. The calluses of 2,4-D/Kin combinations also showed a similar pattern compared with the plant materials. IAA/BA combinations showed a more or less same assay value with the *in vivo* materials (40-60%).

Proteinase inhibitors in plants are closely related to phloem secretions. Phloem differentiation occurs in calluses under the influence of auxin and sucrose[10, 11]. Vascular differentiation also occurs in 2, 4-D/ Kin combinations. However, Oliveira *et al.*[12] observed that production of proteinase inhibitors stops with the conversion of compact callus into friable form.

In conclusion, *C. indica* callus lines were growing faster and showed more Tpi activity than intact plants. Variation of the hormonal composition influenced the growth and Tpi activity. The best medium for Tpi production was MS nutrient medium and the most effective hormonal combination was IBA/BA.

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