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Estimation of the Endogenous Auxins and Cytokinins in Hairy Roots Incited on *Solanum Dulcamara* Plants by Ri Plasmid of *Agrobacterium Rhizogenes*.

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Abstract: Transformed hairy roots induced on *Solanum dulcamara* plants by Ri plasmid of *Agrobacterium rhizogenes* strain 8196 and A4T, exhibited a higher levels of auxin and cytokinin activity as compared with that of non transformed control roots. Endogenous pbytohormones; auxins and cytokinins were determined in an ethyl acetate fractionated extracts using bioassay methods of Hordium coleoptile test for auxins and Cucurbita cotyledonary leaf test for cytokinins. The results obtained gave an excellent explanation for the growth characteristics exhibited by the transfonned hairy roots e.g., rapid growth and intensive branching while growing on MS free phytohormone media. Results obtained agreed with previous reports declaring that Ri T-DNA involves gene encoding certain enzymes sharing in auxin and cytokinin biosynthesis.

Keywords: Agrobacterium rhizogenes, Ri plasmid, hairy roots, plant cell transformation, phytohormones.

INTRODUCTION

Agrobacteria species have been used in plant cell transformation of several species (Tepfer, 1984 ; Hashem, and Davey, 1992 ; Stieger, *et al.* 2004 and Reis, *et al*., 2007). It is known that T-DNA of *Agrobacterium rhizogenes* affects processes of plant development and activates the synthesis of secondary metabolites in transformed plant cells. Application of *Agrobacterium rhizogenes* on higher plants results in hairy roots proliferation from the site of inoculation (Liao and Jhuang, 2007). This was attributed to the transfer of a portion of the bacterial plasmid known as T-DNA to the genome of the plant cell (Chilton, *et al*., 1982 ; Tanaka, *et al*., 2001). Hairy roots induced by Ri plasmid usually exhibiting a vigrous growth and extensive lateral branches when growing on media devoid of phytohormones. Similarly, plants regenerated from hairy roots exhibiting a phenotypic alteration e.g. reducing apical dominance activity of shoot and root growth and also exhibiting an obvious increase in the rooting capability (Tepfer, 1984: Hashem, 1989).

Later studies reported that T-DNA regions of Ri and Ti plasmid genes whose products are capable of synthesizing auxin and cytokinin compounds. For examples (Spano. *et al*., 1988), reported that there were three genes had identified as 10, 11 and 12 on the Ri TL-DNA of *Ag. rhizogenes* were responsible for the increasing of auxin sensitivity of hairy root tissues. This has been followed by reports given before (Epstein. *et al*., 1991) which indicated that, there was a significant increase in the concentration of IAA in transformed callus and induced roots by *Ag. rhizogenes* as compared with the initial concentration in control plants. At the same time (Tinland, *et al*., 1991) has found that there are two genes on Ti and Ri plasmids of *Ag. tumefaciens* and *Ag. rhizogenes* respectively. These two genes coding for enzymes involved subsequently in auxin biosynthesis were identified as (iaam) first gene that coding for tryptophan monooxygenase which convert tryptophan into indole acetamide, and the second gene (iaah) that coding for the indole acetamide hydrolase which convert indole acetamide into indole acetic acid (IAA). Moreover (Camilleri and Jouanin 1991), found that TR-DNA of Ri plasmid in *Ag. rhizogenes* carries two genes identified as auxl and aux2 both are responsible for the auxin biosynthesis in transformed cells.

Concerning cytokinin activity in transformed hairy roots induced by Ri plasmid of *Ag. rhizogenes*, (Liao and Jhuang. 2007), has reported that, an elevated of endogenous cytokinin levels in transformed *Paulownia fortunei* tissues induced by *Ag. rhizogenes*. Estruch, (1991a) also explained that T-DNA regions of the Ri plasmid containing a gene known as rol C is coding for the enzyme cytokinin-beta-glucosidase, which is involved in the cytokinin biosynthesis. The transfer of rol C gene from bacterial T-DNA and its insertion and further integration in the plant cell genome throughout plant cell transformation using *Ag. rhizogenes* might have a drastic effect on growth pattern and development of transformed roots and thereafter regenerated plants. Studying the growth criteria of hairy roots induced as a result of inoculation by *Ag. rhizogenes* showed a more

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vigrous and extensive lateral branches as hairy roots during its growth on MS media devoid of phytohormones (Spano. *et al.,* 1981 ; Guerche, *et al*. 1987 ; Hashem, and Davey, 1992). This alteration in these transformed roots might be preliminary attributed to a modification in the cytokinin and auxin balance in these roots.

In this work we tried to investigate the endogenous phytohormone contents in hairy root tissues induced by using *Ag. rhizogenes* inoculation on *Solanum dulcamara* plants, depending on the bioassayed methods. This method can be performed effectively in standard laboratories and is reproducible and simpler than the other methods depending on expensive instruments for chemical analysis.

MATERIALS AND METHODS

Bacterial strains and culture:

Ag. rhizogenes wild type strain A4T and 8196 were grown on solid APM media, one litre medium was composed of yeast extract (5 g), casamino acid (0.5 g), mannitol (8 g), $(NH4)_2S04$ (2 g), NaCl (3 g), agar (15 g). Cultures were renewed monthly by subculture a single colony in 10 ml liquid APM media (without agar). keeping overnight on a rapid shaker at 28 °C. After the media getting turbid, by using previously flammed inoculating loop, new cultures were streaked, incubated at 28 °C until the colonies started to appear. The culture transferred to the refrigerator for the next month, otherwise they were stored at -20 °C in 80 % glycerol.

Inoculation:

The given axenic shoots (4 cm) in length, *Solanum dulcamara* were decapitated at the top then roots removed using flammed scalpel, then shoot decapitated tops were inoculated using previousely flammed bacteriological loop dipped in bacterial culture previously shaked for 24 hr at (28°C). Loop must bear on its tip a just visible quantity of bacteria to deposits onto the freshly cut tops. Three to four inoculated shoot section were planted vertically in jars containing MS-media (autoclaved) keeping the inoculated tops 2-3 cm well above the medium to minimize the colonization of the medium by *Ag. rhizogenes*. If contamination appear, the inoculum was transferred to jars containing MS-media in addition of 0.5 ug/ml carbenicillin for about one week to get rid of the bacteria then transferred back to MS-media free of antibiotic. All jars contained the inoculated shoots were kept in illuminated room (1000 lux) maintained at 30 °C.

Culture of the induced hairy roots:

Root tips incited at the point of inoculation were exised and rinsed four times with sterile water before being cloned on MS hormone free media supplemented with 0.5 ug/ml carbenicillin. After several transfer without detectable contamination; a culture derived from each decontaminated root tips was established and the growth was monitored daily.

Detection of agropine and mannopine synthesis:

The method used to analyze hairy roots for the detection of agropine and mamiopone was used (Petite, *et al.,* 1983). Two hundreds mg fresh weight of plant material were homogenized with 200 ul of 0.12 N hot HC1, the macerated tissues were then cleared by centrifugation for 10 min at 12.000 xg. 10 ul of the clear extract was potted onto 20x21 cm sheet of chromatographic paper (2 cm apart between different cm from the anode), 1 ul of a mixture of pure agropine and mannopine (0.5 ug/ml) was run simultaneously in a separate lane. The sheet after being wetted uniformly with buffer is subjected to electrophoresis in a buffer consisting of formic acid/acetic acid/distilled water (50/150/800) v/v/v at 300-400 V for 1 hr. Electrophoretogram after being dried for 2-3 hr was stained with silver nitrate 0.25 g $AgNO_3$ dissolved in 20 ml acetone.

Extraction and bioassay for the endogenous phytohormones in the transformed hairy roots:

Three grams of the transformed hairy root as well as normal roots samples were collected and extracted (Mostafa, *et al*., 1990). Extracts of the different samples were simultaneously loaded and developed using : isopropa-nol : ammonia : water (10: 1:1 v/v/v) as a running solvent. The amount of the extract loaded in each case was equivalent to that obtained from 0.25 g dry weight of plant material. The different developed chromatograms were prepared for the various biological tests following the method described by (Hashem, 1984). Bioassays of auxins phytohormone was performed using the straight growth test of Hordeum coleoptile sections (Foda, and Radwan, 1962). Bioassays of cytokinin were carried out using the cotyledonary tissue of Cucurbita seeds (Esashi, and Leopold, 1969). The chromatograms were examined under fluorescence by U.V. lamp and then subjected to some chemical tests for further identification of the nature of the detected growth regulators. Ehrlich's reagent (Powell, 1959), was used as a test for indole compounds, and silver chromate (Reguera, and Ascinov, 1950) as a test for purine compounds having cytokinin activity.

The changes in either lengths of coleoptile sections or area of Cucurbita cotyledonary pieces were expressed as percentage of control and the result of each test was then expressed graphically by a histogram. The results of each test were statistically analysed using the least significance differences (L.S.D.) at 5% level.

RESULTS AND DISCUSSION

Hairy root proliferation:

Three to four weeks after inoculation of decapitated *Solanum dulcamara* axenic shoots by *Ag. rhizogenes* strain A4T and 8196, hairy roots started to proliferate from the point of inoculation in a bush-like, while uninoculated decapitated plants did not exhibit any hairy root proliferation.

Hairy root culture:

Hairy root tips derived from the incited hairy roots cultured on MS media devoid of phytohormones exhibited a rapid growth rate and have showed an extensive branching . On the other hand comparing normal roots derived from the uninoculated plants on the MS media free of phytohormones exhibited a very slow rate of growth and gave no branches.

Detection of opines production in the hairy root culture:.

Production of agropme and mannopine as unusual amino acids are a characteristic features of the real transformed hairy root cultures as confirming the transfer of the T-DNA from plasmids of the *Ag. rhizogenes* and its integration in the genome of the plant cells. Hairy root cultures indued on *Solarium dulcamara* plants by *Ag. rhizogenes* strain A4T and 8196 exhibited the production of mannopine hi the case of strain 8196 and the production of agropine and mannopine in the case of cultures induced by strain A4T.

Endogenouse phytohormone contents in transfo-med hairy roots:

a-Changes in auxins:

Results of the bioassays using coleoptile sections presented in figure (A) showed that, both transformed hairy root extracts either induced by *Ag. rhizogenes* strain 8196 or induced by strain A4T have an increase in the activity levels of auxin substances as compared with the non-transfonned roots extracts. However activity levels of transformed hairy root exracts induced by strain 8196 showed a higher activity levels as compared with those induced by strain A4T. Whereas 8196 transformed root extracts gave 5 promoting zones exhibiting auxiu activity having R_f values : 0.0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-1.0, respectively, with maximum level of auxin activity (70%) expressed as % of increase of coleoptile sections length compared with coleoptile sections that left in water. Colour reaction (table 1) showed that of these 5 zones at least 4 zones that having R_f values: 0.0-0.2, 0.4-0.6, 0.6-0.8 and 0.8-1.0, composed indole compounds as they gave positive colour reactions.

The increase in the activity levels of auxin substances in fractionated extracts derived from transformed hairy root induced by *Ag. rhizogenes* was assumed to be due to the transfere of bacterial Ri T-DNA which might involves genes for auxins biosynthesis, and it was obvious that auxin biosynthetic genes in Ri T-DNA of strain 8196 is more pronounced than those of strain A4T.

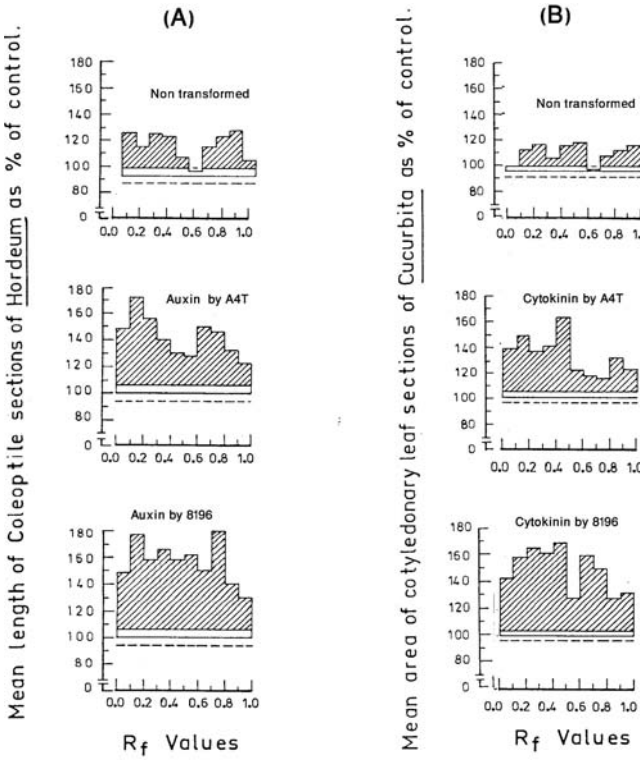
b-Changes in cytokinin and cytokinin-like substances:

Bioassays data using cotyledonary leaf area of Cucurbita for cytokinin activity presented in figure (B) showed that, both transformed hairy root extracts either that induced by *Ag. rhizogenes* strain 8196 or induced by strain A4T have an increase in the activity levels of cytokinin substances as compared with the non-transformed roots extracts. However activity levels of transformed hairy root exracts induced by strain 8196 showed a higher level of cytokinin activity as compared with those induced by strain A4T. Whereas 8196 transformed root extracts gave 5 promoting zones exhibiting cytokinin activity with R_f values : 0.0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-1.0, respectively with maximum level of cytokinin activity (80 %). Colour reaction (table 1) showed that these 5 zones comprised purine compounds as they produced positive colour reactions with reagents testing for purine compounds.

Results of bioassay of fractionated extracts derived from non-transformed control roots showed only 4 promoting zones having Rf values : 0.1-0.3, 0.3-0.5, 0.7-0.9 and 0.9-1.0 respectively, with lower cytokinin activity level (max. 44 %). Colour reaction (table 1) showed that two of these zones that having Rf values: 0.7-0.8 & 0.8-1.0 composed of purine compounds as they produced positive colour reactions, the other two zones predicted to be non purine compounds having cytokinin activities. Similarly, fractionated extracts of hairy roots induced by strain A4T gave 5 promoting zones exhibiting cytokinin activity having Rf valuees : 0.0-0.2, 0.2-0.4, 0.4-0.6, 0.6-0.8 and 0.8-0.1, with maximum increase cytokinin activity level (76 %). Colour reaction (table 1) showed that of these 5 zones at least 4 zones that having Rf values : 0.1-0.3, 0.4-0.6, 0.6-0.8 and

0.8-0.1 comprised indole compounds as they produced positive colour reactions with reagents testing for purine compounds, the first and last zones (0.0-0.1 & 0.9-1.0) were predicted to be non purine compounds having cytokinin activity.

The increase in the activity levels of cytokinin substances in fractionated extracts derived from transformed hairy root induced by *Ag. rhizogenes* was assumed to be due to the transfere of bacterial Ri T-DNA which might involves genes far cytokinins biosynthesis, and it was obvious that cytokinin biosynthetic genes in Ri T-DNA of strain 8196 is more pronounced than those of strain A4T. (max. 45%). Table (2), showed that, these zones comprise purine compounds, where they produced positive colour reactions with reagents testing for purines.



Figures (A & B) : A-Coleoptile test. B-Cotyledonary leaf test, : for fractionated extracts of transformed and non transformed hairy roots induced by Agrobacterium rhizogenes strains A4T and 8196 on Solanum dulcamara plants

Table 1: Chemical tests for detection of indolee and purine compounds in normal and transformed hairy roots induced on Solanum dulcamara plants using Agrobacterium rhizogenes strains 8196 and A4T.

Chemical tests for indole compounds:

Reagents Roots	Normal non-transformed roots	8196-transformed hairy roots	A4T-transformed hairy roots
Ehrlich's	0.7-0.85 +ve	0.0-0.1 5 +ve	0.45-0.6 +ve
		0.45-0.6 +ve	0.65-0.8 +ve
		0.6-0.8 +ve	0.85- 0.95 +ve
		0.8-1.0 +ve	
Ferric/perchloric	0.75-0.85 +ve	0.0-0.2 +ve	0.45-0.6 +ve
		0.45-0.55 +ve	0.6-0.8 +ve
		0.6-0.8 +ve	0.8- 0.9 +ve
		0.85-1.0+ve	

Chemical tests for purine compounds :

Reagents Roots	Normal non-transformed roots	8196-transformed hairy roots	A4T-transformed hairy roots
Silver chromate complex	0.7-0.8 +ve	0. 1-0.25 +ve	0.1-0.25 +ve
	0.85-0.95 +ve	0.35-0.55 +ve	045-55 +ve
		0.7-0.85 +ve	0.6-0.8 +ve
		0.9-1.0+ve	0.85-0.95 +ve

Discussion:

Studying the growth criteria of hairy roots induced as a result of inoculation by *Ag. rhizogenes* showed a more vigrous growth and extensive lateral branches on growing on MS media devoid of phytohormones. In contrast, the non-transformed control roots could not grow on MS media free of phytohormones (Hashem, and Davey, 1992; Spano. *et al*., 1981). This alteration in these transformed roots might be preliminary attributed to a modification in the endogenous phytohormone.

In this work we have investigated the endogenous phytohormone activity in hairy root tissues induced by using *Agrohacterium rhizogenes* inoculation on *Solanum dulcamara* plants, depending on the bio-assay methods. The results showed a highly significant increase in both cytokinins and cytokinin- like substances in these transformed roots compared with the nontransformed one. These results are in agreement with that obtained before (Tinland, 1991) who examined both of the Ti and Ri plasmids of *Ag, tumefaciens* and *Ag. rhizogenes* respectively and he reported that, there are two genes coding for enzymes that involved subsequently in auxin biosynthesis. First gene (iaam) is coding for tryptophan monooxygenase which convert tryptophan into indole acetamide (IAM). Second gene (iaah) was coding for the indole acetamide hydrolase which convert indole acetamide into indole acetic acid (IAA). In addition, (Camilleri, and Jouanin, 1991), also they reportedd that TR-DNA of Ri plasmid in *Ag. rhizogenes* carries two genes identified as auxl and aux2 both were responsible for the auxin biosynthesis in transformed cells. Later on Lutova, and Pavlova, (1999) then Bais, and Ravishankar, (2003) and recently, Thimmaraju *et al*., (2008) reported an increase in the auxin content in the transformed tissues. Concerning, cytokinin contents in transformed hairy roots induced by Ri plasmid of *Ag. Rhizogenes* (Liao and Jhuang. 2007) reported an elevated endogenous cytokinin levels in transformed petunia tissues induced by *A. rhizogenes* and *A. Tumefaciens.* Likewise, Estruch, J.J. (1991a) clarified that T-DNA regions of the Ri plasmid contaming a gene known as rol C is coding for the enzyme cytokinin-beta-glucosidase, which is involved in the cytokinin biosynthesis.

The prospects of such increase in the activity levels of endogenouse phytohonnones in transformed root cells and derived regenerated plants due to insertion of Ri T-DNA in plant cell mediated by *Agrobacterium rhizogenes* would offer a several advantages for improvement of the the agricultural crops For instance, the increase of auxins might increase rooting capability for plant cuttings and grafting (Druart, and Gruselle, (2007) which can be used in vegetative propagation of plants. Whereas transformed cells are capable to synthesize their endogenous phytohormone automonously instead of adding external auxins. Moreover, increased auxins of root cells might support their active absorption which in turn unable plants to tolerate drought and salt stress, this will be taken into consideration in future work. Similarly, increase in the levels of endogenouse cytokinin of transformed root cells might induce an increase in the rate of cell division of root cells which may establish a well developed root system.

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