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ORIGINAL ARTICLE

Biochemical studies on Crown Gall Disease in *Cantharanthus roseus* induced by *Agrobacterium tumefaciens*

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ABSTRACT
Biochemical resource manipulation is typical of bacterial-plant gall interactions. Catharanthus roseus (Vinca rosea) was the organism of choice to demonstrate the effects of tumours induced by the bacterium Agrobacterium tumefaciens. The choice of this particular subject is based on the fact that the plant is known to possess several important medicinal properties, and is used extensively in the field of anti-cancer drug production. The pathogen chosen is of great relevance in the field of biotechnology. Analytical procedures were carried out to demonstrate the variations of several parameters such as auxins, cytokinins, flavonoids, β-carotene and total protein. Along with the above mentioned parameters, the activity of the antioxidant enzyme peroxidase was also determined. As the various parameters analyzed provide a wholesome measure to determine the extent of damage caused by the pathogen, the results thus obtained helped us evaluate the effects of tumors on various facets of the plant growth.
***Keywords**: Auxins, Beta-carotene, Cytokinins, Flavonoids, Peroxidase, Tumour*

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INTRODUCTION

Crown gall is a plant disease that affects Gymnosperms and Dicotyledons resulting in the formation of anaplastic tumors. The causative organism, *Agrobacterium tumefaciens* transforms the host cells inducing unlimited growth, which is largely independent of morphogenetic restraints of the host plant. The crown gall resembles neoplastic growth encountered by both animals and plants.

Catharanthus roseus belonging to the family *Apocynaceae* is an important plant used traditionally in a number of countries for the treatment of various diseases (such as diabetes, hypertension, cancer, menstrual irregularities etc.). It is a rich source of dimeric alkaloids namely, vinblastine and vincristine used extensively in the treatment of human neoplasma. The monomeric alkaloid ajmalicine has found broad application in the treatment of circulatory diseases, especially in the relief of circulatory obstruction of normal cerebral blood flow. Sulphated vinblastin is used to treat Hodgkin's disease, lymphocarcoma, choriocarcinoma, neuroblastoma, carcinoma of the breast, lungs and other organs [6].

As part of an investigation of the pathological plant growth induced by the crown gall organism *Agrobacterium tumefaciens* it appeared desirable to study the nature and biochemistry of both diseased and contiguous tissue.

MATERIALS AND METHODS

The gall forming organism *A. tumefaciens* (MTCC No. 609) was obtained from the Institute of Microbial Technology, Chandigarh, India in the lyophilized form. The organisms were rejuvenated by inoculation into Nutrient broth under aseptic conditions followed by streaking onto Nutrient agar. Several sub-cultures of the bacterium were maintained to maintain viability. On obtaining dense growth on the Petri plates, the bacterium *A. tumefaciens* was taken in vials containing 0.85% saline solution and inoculated into the upper internodes of the twelve-week old test plants. A sufficient amount of gall tissue for analysis was produced in three weeks of inoculation. Simultaneously with the collection of galls, sections of the

stem immediately above and below the galls were taken to furnish samples of contiguous tissue. Healthy non-inoculated plants were used as control.

Indole-3-acetic acid levels were determined according to Mahadevan and Chandramohan [15]. 1 g of fresh leaf and stem samples of the control and infected plants were macerated in 30 ml ethanol and allowed to stand for 24 h. The homogenate was passed through Whatman filter paper and the resultant filtrate was concentrated by evaporation and dissolved in methanol. Indole-3-acetic acid in methanol was determined with Salper reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid). Absorbance was read at 535 nm using a spectrophotometer. The amount of indole-3-acetic acid was calculated from the standard curve.

Cytokinin was extracted by maceration in Mc Ilvaine's buffer (pH 5.6) and suspended in 24 ml ethanol at 4°C overnight. The homogenate was then centrifuged at 2000 rpm for 5 min and the resulting supernatant was reduced to ~ 10 ml by evaporation. The pH was adjusted to 2.9 using 1N HCl and mixed well with equal volume of diethyl ether to remove contaminating chlorophylls, auxins and gibberellins. The pH of the aqueous phase was adjusted to 7.8 with 1N NaOH. Extraction was continued with 5 ml portions of n-butanol 5 times in a separator funnel. The butanol layers were pooled together and evaporated to dryness under reduced pressure. The resulting residue was dissolved in 2 ml of buffer and used for estimation. For estimation, 1 ml of the sample was made up to 4 ml with Mc Ilvaine's buffer and the absorbance at 250 nm was noted. Kinetin was used as a reference standard (10mg/ 100ml).

β-carotene was extracted according to the method of the Association of Official Analytical Chemists [1]. Leaf and stem tissue (1 g each) of the control and infected plants were homogenized with 10 ml methanol and then passed through a Whatman filter paper. The fluid phase was further filtered (repeatedly) until it was pigment-free. The filtrate thus obtained was portioned with an equal volume of diethyl ether. Water was added in minimal amounts to allow distinct separation of the two layers. The ether layer containing carotenoids was evaporated and the resultant residue was dissolved in minimal amount of ethanol. 10 ml of 60 % aqueous KOH was added in small increments to enable saponification and thereby remove contaminating chlorophyll and lipids. This also serves to cleave esterified carotenoids. The mixture was then subjected to boiling for 10 min. An equal amount of water was added and extraction was carried out using diethyl ether. The ether was evaporated off under reduced pressure. The residue obtained was dissolved in a minimal amount of ethanol and quantitated spectrophotometrically at 436 nm using β-carotene (100 µg/ml) as standard.

Flavonoids were extracted by macerating 1 g of plant tissue in 10 ml methanol:water mix (4:1). The filtered extract was partially evaporated and acidified with ~ 6 drops of 2M H_2SO_4. This was followed by addition of 10 ml chloroform:water (3:1). The chloroform layer separating out is allowed to remain exposed to the air for about 12 h. On complete evaporation, the residue containing flavonoids was dissolved in a minimal amount (~ 0.5 ml) of methanol and quantitated using aluminium chloride colorimetric method with quercetin as standard [24].

Guaiacol peroxidase (POX, E.C. 1.11.1.7) Fresh tissue were homogenized with pre-chilled 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM β-mercaptoethanol and 1mM EDTA using pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. Guaiacol peroxidase activity was measured in a reaction mixture of 3.0 ml consisting of 50 mM phosphate buffer (pH 7.0) containing 20 mM guaiacol, 10 mM H_2O_2 and 100 µl enzyme extract [9]. The formation of tetraguaiacol was followed by an increase in A_{470} nm (ε = 26.6 mM^{-1} cm^{-1}). One unit of peroxidase is defined as the amount of enzyme needed to convert 1 µmol of H_2O_2 min^{-1} at 25°C.

Total proteins were extracted from leaf and stem tissue of the control and infected plants by maceration using hot 80% ethanol. The homogenate was centrifuged at 3000 rpm for 10 min and the resulting pellet was re-suspended in 5% TCA and placed in an ice bath for 20 min. The tubes were subjected to centrifugation again and the supernatant was discarded. The pellet was re-suspended in hot absolute ethanol and centrifuged. The final pellet was used for determination of protein content according to the method of Lowry et al., [14].

Statistical analysis The experiment was performed using a randomized design. All data are expressed as means of triplicate experiments. Comparisons of means were performed using PrismGraph version 3.02. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by lowest standard deviations (LSD) test. Comparisons with $P \leq 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

The production of the phytohormones auxins (e.g. indole-3-acetic acid-IAA) and cytokinins are important virulence factors for the gall-forming phytopathogenic bacteria. An increase in the levels of both auxin

and cytokinin was seen in *C. roseus* infected by *A. tumefaciens* (Table 1). Experiments on crown gall have indicated the involvement of *tms* loci encoded enzymes in a two step pathway for the production of auxin from tryptophan. The *tms1* gene encodes tryptophan monooxygenase that converts tryptophan to indole-3-acetamide [20, 23] that is subsequently converted to auxin IAA by amidohydrolase, the product of the *tms2* gene [21].

Table 1: Quantitative estimation of biomoleculas in uninfected and infected gall tissue

Biomolecule assayed	Tissue sample	
	Uninfected	Infected
Auxin*	680.4 ± 12.3	1020.7 ± 18.6
Cytokinin*	115.3 ± 8.1	233.8 ± 6.2
β-carotene*	1050.8 ± 21.6	460.6 ± 6.6
Flavonoids*	29.3 ± 3.2	87.4 ± 4.9
Peroxidase#	5.9 ± 0.2	7.7 ± 0.5
Total protein*	440.8 ± 9.6	2720.1 ± 18.4

*** µg/g of fresh weight tissue, # IU/g fresh weight tissue**

In addition to the *tms* genes, T-DNA of *A. tumefaciens* also encodes *tmr* gene for the synthesis of isopentenyl transferase required for the production of cytokinin *iso*-pentenyladenosine 5'-P [2,7]. Similar increase in auxin and cytokinin levels was reported in olive and oleander knot disease by the causal organisms *Pantoea agglomerans* and *Pseudomonas savastanoi* respectively [8]. Tumorigenesis in olive knot disease requires the continuous presence of the causative organism for the production of auxin and cytokinin; while, in crown gall induced by *A. tumefaciens,* the Ti plasmid containing the phytohormone biosynthetic genes alone can suffice for tumour induction and maintenance [11].

In vascular plants, the phenylpropanoid pathway results in the production of flavonoids, a diverse collection of compounds involved in pigmentation, herbivory defense, and plant–microbe interactions. A 3-fold increase in flavonoid content was seen in tissues of *C. roseus* infected by *A. tumefaciens* (Table 1). Flavonoids are known to prevent oxidation and basipetal transport of auxin, which is then diverted to lateral and apical directions resulting in the development of circular vascular bundles [3,4]. This induces own transport systems and enhances local nodule growth [13,16]. An increase in the activity of chalcone synthase, the first committed enzyme for flavonoid biosynthesis and the product of the T-DNA-located gene*6b* has also been reported in the maintenance of high auxin/cytokinin concentrations [5]. Futhermore, an accumulation of 7,4′ dihydroxyflavone and formononetin was seen in tissue areas of GUS-labelled chalcone synthase selectively in crown galls of transgenic *Trifolium repens* [19].

An overall increase in protein levels (Table 1) indicates that the diseased state does not essentially inhibit the protein synthesis. Another view would state that certain proteins may also be synthesized, which may be seen as control measure for adaptation to the tumour state. Enhanced peroxidase activity (Table 1) in infected tissue might be caused by its involvement in the regulation of IAA content [17]. An inverse relationship of peroxidase activity and growth has been attributed to its ability to oxidise IAA [22]. The biosynthetic pathways of growth regulators in crown galls differ from the corresponding pathways of uninfected plant tissue [12]. This increase in activity can be extrapolated to cell line transformants and may be used in the commercial production of peroxidase. The infected tissue was found to have > 50% decline in β-carotene levels (Table 1) indicating a possible role in scavenging of free radicals generated on account of biotic stress [10].

CONCLUSIONS

Biochemical studies on crown gall at the initial stages have assisted in the understanding of biochemical parameters needed for the induction, establishment and maintenance of crown gall in *Agrobacterium tumefaciens*. The decrease in β-carotene levels and an increase in flavonoid levels help in the induction of tumor. The surge in hormonal activity (auxins and cytokinins) appears to be a vital factor for successful establishment of the tumour. Methods for the regulation of these hormones in susceptible plant hosts may help in preventing the spread of the disease.

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