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Research Article

Understanding the effect of seed pathogenic bacteria in release of conjugated forms of endogenous hormones from the seed of *Cajanus cajan* by Indirect ELISA

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ABSTRACT

Bacteria as plant pathogen are known to attack diverse crops of economic significance. Many of disease symptoms caused by phytopathogenic microbes appear to reflect the changes in plant hormones levels. In this study, gram negative bacteria, inhibiting seed germination were isolated from the seeds of *Cajanus cajan* and maintained on Nutrient agar medium. This bacterium, identified on the basis biochemical tests and 16s rDNA sequencing as *Pantoea agglomerans.* To study the role of pathogenic bacteria in food mobilization, five hundred mg of dry seed powder was treated with different concentrations of N-broth (1ml to 5 ml bacterial culture) for 48 h. Control was prepared with uninoculated N-broth. Endogenous hormonal levels of treated and untreated seed powder were estimated by indirect ELISA. To estimate endogenous hormonal concentration, antibodies against IAA, PAA, KiN, GA, and ABA were raised in rabbits. IAA, PAA, KiN, GA, ABA were conjugated with BSA and injected to the rabbits via intramuscular routs. Antibodies against each plant growth hormones was purified and used for estimation of free and conjugated forms. It was observed that bacteria have released bound IAA, PAA, KiN, GA, and ABA from dry seed powder significantly. These results were confirmed by comparing the data with control. The probable mechanism of pathogenic bacteria in hydrolyzing conjugated forms of all hormones in *C. cajan* is discussed.

Key Words*: Pantoea agglomerans,* IAA, PAA, KiN, GA and ABA

INTRODUCTION

Pigeon pea (*Cajanus cajan*) is an important pulse crop of India, which imports 8,000 to 10,000 metric tons of peas per year and it is a rich source of proteins, carbohydrates, and minerals (Salunkhe *et al.,* 1986*)*. Diseases and pests are major constraints to legume production, especially in the tropics and subtropics. Plant disease is responsible for significant losses of worldwide crop production every year and, thus has a major impact on the world's agricultural yield (Nölke *et al.,* 2004). The numbers of bacteria are generally associated with plants, which may be beneficial or disadvantageous. Plant hormones can be considered imperative signals in establishing associations between plant and microbes. Many of disease symptoms caused by phytopathogenic microbes appear to reflect the changes in plant physiology.

Many pathogens disturb the hormone balance in plants by either releasing plant hormones themselves, or by triggering an increase or a decrease in synthesis or degradation of hormones within the plant. Several genes of bacterial origin are able to change phytohormones content and their activity in plant have been characterized, including genes whose products can synthesize and modify phytohormones and / or hydrolyze phytohormones conjugates (Spena *et al.,* 1992). Alterations to the biological activities of phytohormones can result in modification of plant physiological and development processes. Microbial organisms that are pathogen or symbionts of plant may helpful as neutral models for studying such modification.

Phytohormones are important in seed germination process. However, reports on pathogen induced changes in hormonal levels are rather scanty. Taking these points into consideration, in present study, pathogenic bacteria inhibiting seed germination were isolated and identified by 16s rDNA sequencing. Free and bound forms of endogenous hormones released by seed pathogenic bacteria were estimated. Since phytohormones synthesized in small quantities a sensitive and accurate method of an enzyme immunoassay was developed for hormonal analysis in plant tissue. In addition this technique does not require a well equipped laboratory. Antibodies were raised against each plant growth hormones and endogenous levels of each hormone were estimated by more sensitive and specific immunoassay i.e indirect ELISA.

MATERIALS AND METHODS

Infected seed material

Certified seeds of *Cajanus cajan* ($B.D.N_2$) *were* purchased from the local market, Rajkot. Equal sized seeds were screened, washed with tap water for 2-3 times and soaked in distilled water for 3 h. Seeds were transferred in wet filter paper for germination and kept in dark for 48 h. Non germinated and bacterial contaminated seeds were selected. Bacteria were isolated and maintained on nutrient medium.

Identification of pathogenic bacteria from the non-germinated seeds

The strain (SU01) was identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergey's Manual of Determinative Bacteriology (Table 1). Isolated colony was selected from the N-agar medium and inoculated in N-broth medium. Bacteria were incubated at 37^0C and after 24 h different concentrations of organisms were used for the assay.

Viable count

The number of bacterial cells per ml culture was counted by viable count method.

Identification by16S rDNA sequencing

DNA extraction

Bacterial cells were pelleted by centrifugation, resuspended in 500 µl of 10 mM Tris-EDTA buffer, and treated with 30 µl SDS (10% W/V), 2 µl Proteinase K (10mg/ml). It was mixed well and incubated for 1 h at 37^0C. Then after 20 µl of Cetyltrimethylammonium bromide (10%, W/V) and 100 µl of NaCl (5 M) were added, and incubated for 10 min at 65°C. DNA was purified by two 1:1 extractions in which we used (i) Chloroform: Isoamyl alcohol (24:1) (ii) Phenol: Chloroform: Isoamyl alcohol (25:24:1) and then was precipitated with isopropanol, washed with ethanol (70%), and dissolved in Tris-EDTA buffer. The quality and concentration of the DNA was confirmed by measuring optical density 260 /280 nm ratio.

16S rDNA gene amplification

The 16S rDNA gene was amplified using universal primer pair 8F (5-AGA GTTTGATCCTGGCTCAG-3') and 1525R (5'-ACGGCTACCTTGTTACGACTT-3'), 946F (5'-CCCGCACAAGCGGTGGA-3') and 1389R (5'-ACGGGCGGTGTGTACAAG 3'). DNA was amplified in a total volume of 25 µl. The reaction mixture contained 2.5 µl 10X buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Trion X100), 1.5mM $MgCl_2$, 200 µM each dNTP, 10 µM primer and 1U of *Taq* DNA polymerase, 200 ng bacterial DNA. Profile of PCR was: initial denaturation 95^0C - 5min: followed by 35 cycles: denaturation 95^0C - 30 s , annealing 52^0C - 45 s, extension 72^0C - 2 min and final extension 72^0C - 12 min. Amplified DNA fragments were separated by electrophoresis through 1.5% Low melting agarose gel. DNA fragments were eluted from low-melting temperature agarose gels. The band of interest is excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at -20^0C, and then melted. TE-saturated phenol was added to the melted gel slice and the mixture was again frozen and then thawed. After this second thawing, the tube centrifuged and the aqueous layer transfered to a new tube. The DNA was concentrated by ethanol precipitation.

Sequencing of 16S rDNA gene

The eluted PCR products were sequenced using a Big Dye Terminator V 3.1 Cycle Sequencing Kit using ABI 3130 genetic analyzer. The sequencing reaction required 1 µl of Premix, 10 pmol of sequencing primer and 200 ng of the PCR product template in a total volume of 10 µl. 16S rDNA partial sequence was determined using 8F and 1525R sequencing primers. All sequencing reactions were performed using the Veriti ™ Thermal Cycler with 45 cycles of denaturation (95°C, 30 s), annealing (52^0C, 20 s) and extension (60°C, 4 min).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been submitted to the NCBI GenBank database (Accession number SU01 strain-JF501472).

Raising of antibodies against PGRs

Preparation of PGR-BSA conjugate

PGRs-BSA conjugate were prepared according to Bhatt *et al.,* (2008). IAA (52.3mg) or PAA (100mg) or GA (106mg) or ABA (132mg) or KiN (10mg) was dissolved in 2 ml of DMF and reacted with 75-µM tri-n-butyl amine and the solution was cooled to (0^{o}C), 40 µl isobutyl chlorocarbonate was added and incubated for 8 min at room temperature. This reaction mixture was added with constant stirring to 421 mg BSA dissolved in 22 ml of DMF: water (1:1, v/v) and 420 µl 1M NaOH. After 1 h incubation at 0^{o}C another 0.2ml of 1M NaOH was added and stirring was continued for 5 h. The mixture finally dialyzed against 10 % DMF for 24 h and against distilled water for 4 day.

Immunization

Two rabbits for each PGR were immunized by intramuscular rout according to Thaker, (1995). Antigen was prepared by mixing PGR-BSA conjugate thoroughly with equal volume of adjuvant and injected into rabbits. Booster injections were given periodically to raise the titer. Rabbits were bled periodically, serum was separated and antibodies were purified by ion exchange chromatography using DEAE cellulose cake. Purified antibodies of each hormone were stored in freeze before used.

Extraction of Hormones

For the extraction of hormones, five hundred mg of dry seed powder was taken and mixed with five different concentrations of bacterial broth (1.0 to 5.0 ml culture). The mixture was incubated at 37^{0}C for 48 h in dark. Hormones were extracted with 80% methanol containing mg/ml ascorbic acid. Samples were centrifuged at 10,000g for 10 min. Supernatant with methanol was collected and kept in dark for evaporation. From the evaporated samples final volume (10 ml) was prepared with phosphate buffer saline, pH 7.2.

Estimation of endogenous hormones

Endogenous level of hormones viz. IAA, PAA, KiN, GA and ABA were estimated by a comparatively more sensitive and specific technique i.e. indirect ELISA. PGR-casein conjugate (300 µl) was coated on ELISA plate and incubated for overnight at 4^{o}C, followed by washing with PBS-T. The next step involved was blocking of free protein binding sites of well with egg albumin and incubated for 1 h at 37^{o}C. Antibodies against PGRs mixed with samples, were coated and incubated for 3 h at 37^{o}C. Finally, the plate was coated with anti Rabbit IgG, tagged with peroxidase and the color was developed using O-phenylene diamine as a substrate. The reaction was terminated by addition of 6N sulfuric acid (50 µl). After each coating, the ELISA plate was washed thoroughly with PBS containing 0.05 % Tween-20. The color developed was measured at 490 nm by ELISA Reader (µ Quant, Biotek, USA). Assay for each hormone was performed thrice and mean value was calculated.

RESULTS AND DISCUSSION

In this study, bacteria isolated from the infected, non-germinated seeds of *C.cajan* were gram negative, rod shaped cells, having small, round, smooth textured, transparent and slightly yellow-pigmented colonies. The results of biochemical tests revealed that seed pathogenic organism isolated from the infected seeds is *Pantoea agglomerans,* belong to Enterobacteriaceae family (Table 1).

They are facultative anaerobes producing acid from sugars by fermentation. Most members of this family reduce nitrate, form catalase, and are negative for indole production. *Pantoea* species are found in natural environment and isolated from plants, flowers, seeds and vegetables and from a wide variety of environmental sources, such as sewage, soil and vegetables (Richard, 1984). Further bacteria identified by 16S rDNA sequencing. Sequences of 16S rDNA from strain SU01, was shown to have a 93 % similarity with *Pantoea agglomerans* by BLAST analysis. The Accession number derived for this sequence was JF501472. Bacteria from the genus *Pantoea* have become increasingly important plant pathogens around the world. Many plant pathogenic *Pantoea* species are seed borne and seed transmitted such as *P. agglomerans* in cotton (Medrano and Bell, 2006). *P. agglomerans*, is also causes the diverse disease, including gall, rots, wilt, leaf blights, necrosis, dieback and bulb rot (Grimont and Grimond, 2005; Edens *et al.,* 2006). In this work antibodies were raised against each plant hormone i.e., IAA, PAA, KiN, GA, and ABA. Estimation of hormones in plant tissues a sensitive and accurate

method of an enzyme immunoassay was developed. The advantage of immunoassay for hormone analysis is the ability to use crude plant extract. In addition, this technique does not require a well equipped laboratory; but it required long time of immunization as all plant hormones are heptans. The antibodies raised with the different hormones using pure commercial chemical, showed hardly any cross-reactivity with each other (Table 2) and a great number of samples can be tested in much less time for all group of plant hormones.

Table 1: Results of Biochemical tests

No.	Biochemical tests	Result
1	Sugar Fermentation	
	Glucose	+Ve
	Lactose	+Ve
	Xylose	+Ve
	Manitol	+Ve
	Sucrose	+Ve
	Maltose	+Ve
	Ribose	+Ve
	Sorbitol	+Ve
	Myo-inositol	+Ve
	Galactose	±Ve
2	M.R	+Ve
3	V.P	+Ve
4	Catalase	+Ve
5	Strach hydrolysis	-Ve
6	Gelatin hydrolysis	-Ve
7	Casein hydrolysis	+Ve
8	Reaction in milk	Acidic
9	Indole Production	-Ve
10	H_2S Production	-Ve
11	NH_3 Production	+Ve
12	Reduction of Nitrate	+Ve
13	Urea hydrolysis	+Ve
14	Citrate Utilization	+Ve
15	Tween 80 hydrolysis	
16	Salt Tolerance	
	0 %	+Ve
	1%	+Ve
	2%	+Ve
	3%	+Ve
	4%	+Ve
	5%	+Ve

In plant tissue, three statuses of hormones are reported as (I) free (II) conjugated and (III) oxidized forms. Free forms are considered to be readily available for growth. Conjugated forms either participate in hormone transport or remain as storage pool, or function as hormone protectors from enzymatic attack (Gokani and Thaker, 2002). Conjugation, the binding of low molecular weight compounds to the hormone, represents a mechanism to regulate the cellular level of 'active' hormones by generating products with little biological activity (Brozobohaty *et al.,* 1994). Thus conjugated forms of hormones are most important in seeds, which may hydrolyze during the process of seed germination. The germination process involves the metabolic awakening of the latent seed and resumption of developmental processes. That storage pool is disturbed by pathogenic bacteria, is well established (Chou *et al.,* 1996). Thus, the released of conjugated hormones in treated seed powder in this work support the view that bacteria might have influenced on the hormonal pool by cleavage of conjugated form. The percentage release of endogenous level of each hormone by bacterial activity is presented in Table 3. Control sample shows free forms of hormones in the seed powder.

One ml N-broth contained 506.3 x 10^4 of bacterial cells. By increasing the bacterial concentration, the amount of released hormone also increased and it became stable at higher concentration. By specific antibodies, it is very easy to differentiate two natural auxin; IAA and PAA. IAA was released gradually with increase in amount of bacteria. The percentage of released IAA was 67.88% with 5 ml bacterial concentration. In comparison to other hormones IAA is released in higher amount. IAA is stored in conjugated forms that are mostly considered to be inactive. On average, 95% of all IAA in a plant is conjugated to storage forms, (Cohen and Bandurski, 1982). The amount of PAA released by bacteria was 41.34%. Maximum PAA was released with 4 ml bacterial concentration. The presence of higher cytokinin activity in the endosperm than in the embryo during the first 24 h after germination suggests that the endosperm may supply cytokinins until the embryo is able to synthesize its own cytokinins. Kinetin was released in lower amount and the percentage of KiN was 23.93%. Gibberellic acid (GA) was released gradually by bacteria. Maximum amount of GA was obtained with 3 ml of bacterial broth and later there was no increase. The percentage of released GA was 48.12%.

Table 2: Cross reactivity test for the antibodies raised against each PGR with structurally related natural and synthetic components

% Reactivity with antibodies against

	IAA-BSA	PAA-BSA	GA-BSA	ABA-BSA
IAA	100	0.5	0.2	0.1
PAA	0.3	100	0.1	0.05
GA	0.8	0.2	100	0.1
ABA	0.8	0.5	0.9	100
IBA	1.0	-	0.9	0.2
IPA	0.7	0.5	-	0.3
ICA	0.9	1	0.8	0.7
IAN	0.3	1.5	0.2	0.9
NAA	0.8	0.1	0.9	0.1
2,4-D	0.4	5	1.0	0.05

Table 3: Percentage release of endogenous hormonal level with different concentration of bacteria

Concentration (ml)	**IAA** µg g^{-1}seed dry wt	**PAA** µg g^{1}seed dry wt	**KiN** µg g^{-1}seed dry wt	**GA** µg g^{-1}seed dry wt	**ABA** µg g^{-1}seed dry wt
Control	42.93	45.2	44.48	80.1	103.3
1	89.06	60.53	41.97	140.77	151.2
2	84.11	66.93	40.61	152.4	154.3
3	70.94	69.06	58.48	154.4	181.5
4	117.2	77.06	41.27	140.4	217.4
5	133.66	68.66	35.55	145.4	156.5
Percentage	**67.88%**	**41.34%**	**23.93%**	**48.12%**	**52.48%**

Note: Maximum µg released PGRs by *Pantoea agglomerans* activity was considered as 100% and percentage release of endogenous levels was calculated with comparison to control.

Furthermore, amount of released bound gibberellins were also higher with increasing bacterial concentration as compare to control.This indicates ability of bacteria in metabolizing seed nutrients. With increasing bacterial concentrations, more amount of endogenous ABA is released. The ABA was released in 52.48%. There was a gradual increase in ABA level with increase in the bacterial concentration where 4 ml showed maximum release and declined there after. This shows the saturated level of ABA in mature seed. It is well known that ABA does not inhibit initial imbibitions of water needed for initial embryo extension growth, but it inhibits the post germination extension growth of the radical (Meurs *et al.,* 1992).

In general almost 23.93% (KiN) to 67.88% (IAA) of phytohormones were released with bacterial activity. Earlier work on soybean with *Enterobacter agglomerans* by Chou *et al*. (1996) showed hydrolysis of conjugated forms of IAA. The presence study with selective and sensitive immunoassay, lead to a conclusion that these bacteria can also hydrolyze other conjugate phytohormones besides IAA. However, further study requires answering whether all phytohormones are conjugated with similar storage protein molecule or the bacteria have different hydrolytic properties for each conjugated phytohormones.

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Abbreviations*:* 2, 4-D; 2, 4-Dichlorophenoxy acetic acid, ABA; Abscisic acid, BSA; Bovine serum albumin, ELISA; Enzyme Linked Immunosorbent Assay, DEAE cellulose; Diethylaminoethyl cellulose, DMF; Dimethyl formamide, GA; Gibberellic acid, IAA; Indole-3-acetic acid, IAN; Indole-3-acetonitrile, IBA; Indole butyric acid, ICA; Indole-3-carboxylic acid, IPA; Indole-3-propionic acid, KiN; Kinetin, NAA; Naphtalene acetic acid, PAA; Phenyl acetic acid, PBS; Phosphate buffer saline, PGR; Plant growth regulator

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