INHIBITION OF POLYGALACTURONASE ACTIVITY OF RICE PATHOGENS BY THE OXIDIZED PRODUCTS OF CHLOROGENIC ACID

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PENETRATION and invasion of host tissue by a pathogen is substantially enhanced either by the production of cell wall degrading enzyme(s) or toxin(s) or by the synergistic action of these enzyme(s) and toxin(s). Production of pectinolytic enzymes by phytopathogenic microorganisms has been reported widely. Though the exact role and mode of action of these enzymes in pathogenesis are still subjected to discussion[1], polygalacturonase, one of the principal cell wall degrading enzymes is well known for its ability to macerate the host tissue[2].

Inactivation of pectic enzymes by polyphenols and their oxidized products, (the quinones) has been earlier reported[3-7]. In the present work the effect of chlorogenic acid[8] and its oxidized products on the PG of *Rhizoctonia solani* and *Acrocylindrium oryzae*, the rice sheath blight and rot pathogens respectively, was studied.

R. solani (Manian[9], ATCC 48502) and *A. oryzae* isolated from diseased rice plants by the authors were used for the production of PG. PG production was carried out in the modified Czapek-dox broth[10] with casein hydrolysate 0.2%, sodium polypectate 1.0%, pectin 1.0% and glucose 0.1% serving as the organic constituents. The pH of the medium was adjusted to 7.0. Erlenmeyer flasks (250 ml) with 50 ml medium each were inoculated with 1 ml mycelial suspension (*R. solani*) or 1 ml of spore cum mycelial suspension (*A. oryzae*). Cultures were incubated on orbital shaker at 28 ± 1°C. Cultures for enzyme assay were harvested on 5th and 3rd days for *R. solani* and *A. oryzae* respectively as optimal amounts of PG activity was detected in the culture filtrates (CF) on these days in the preliminary study. CF was centrifuged at 12,000 g for 15 min, followed by filtration through Whatman No. 1 filter paper. The clear supernatant was dialyzed against cold glass distilled (GD) water for 48 hr with a minimum of 3 changes of water. The retentate served as the enzyme source.

All procedures followed for the assay of PG before and after incubation with chlorogenic acid and its oxidized products were the same for both the pathogens. Viscometric assay of polygalacturonate splitting enzyme was made in Fenske-Ostwald viscometers. The reaction mixture for the enzyme assay contained 2 ml of 2% sodium polypectate, acetate buffer (2 ml pH 5) and the enzyme (1 ml). Boiled enzyme preparation served as control. Activity was recorded as viscosity reduction units (VRU) defined as $100/t_{50}$ where t is the time in min required for 50% reduction in viscosity of the reaction mixture at 35°C by 1 ml of enzyme.

Formula followed for calculating the reduction in viscosity

$$\frac{t_{eo} - t_e}{t_{eo} - t_w} \times 100$$

where t_{eo} is the flow time for boiled enzyme
t_e is the flow time for the enzyme
t_w is the flow time for water

Preparation and incubation of phenol with PG

Chlorogenic acid dissolved in 0.1 M phosphate buffer, pH 7.0 was used for incubation with PG. The incubation mixture consisted of 2.5 ml enzyme, 1 ml of phenol in buffer and 1.5 ml of phosphate buffer (0.1 M, pH 7.0). Phenol content of the incubation mixtures was adjusted to a final concentration of 1.0, 2.0, 4.0 and 6.0 μm/ml. After an incubation period of 1 hr the mixtures were assayed for PG activity as described elsewhere.

For studies with the oxidized products of chlorogenic acid, phenolase from potato peels was extracted, partially purified according to Patil and Zucker[11] and was used. The incubation mixture had 2.5 ml enzyme, 1 ml phenol in buffer, 0.5 ml phenolase preparation and 1.0 ml of phosphate buffer. All these were added simultaneously. Final concentration of the phenol in the incubation mixtures was adjusted to 0.5, 1.0, 1.5 and 2.0 μm/ml. After 1 hr incubation the test mixtures were assayed for PG activity. All incubations were carried out at 28 ± 1°C.

The rice sheath blight and rot pathogens *R. solani* and *A. oryzae* produced good amounts of PG. PG activity of *R. solani* was 16.7 VRU and that of *A. oryzae* was 28.6 VRU on the 5th and 3rd days respectively. Chlorogenic acid even at relatively high concentration (6.0 μm/ml incubation mixture) did not inhibit the PG activity of either of the rice pathogens. Incubation of PG of *R. solani* or *A. oryzae* with the phenolase preparation (which was used for oxidizing the polyphenol) alone did not reveal any inactivation of PG by phenolase.

a) *Effect of the oxidized products of chlorogenic acid on the* PG *of R. solani*

Quinones of chlorogenic acid inhibited the PG activity of *R. solani* to a maximum of 46.9% at the concentration of 1.5 μm of the phenol/ml of the incubation mixture. Table 1 shows the percentage inhibition of PG activity by the oxidized products of chlorogenic acid at different concentrations of the phenol.

b) *Effect of the oxidized products of chlorogenic acid on the* PG *activity of A. oryzae*

A maximum of 53.7% repression of the PG activity of *A. oryzae* was effected by the oxidized products of chlorogenic acid at the concentration of 1.5 μm/ml of the incubation mixture. Table 2 shows the effect of oxidized products of chlorogenic acid on the PG activity of *A. oryzae* at various concentrations of the phenol. However, phenol concentrations both above and below 1.5 μm/ml showed less inhibition on the PG activity of *R. solani* as well as *A. oryzae*.

Table 1 *Effect of oxidized products of chlorogenic acid on* PG *activity of R. solani.*

Concentration in μm/ml	Enzyme activity (units/ml) Phenol + phenolase	Enzyme activity (units/ml) Original*	Inhibited activity (units/ml)	Percentage inhibition
0.5	13.42		3.24	19.4
1.0	11.58		5.08	30.5
		16.7		
1.5	8.85		7.81	46.9
2.0	10.25		6.41	38.5

* PG activity of the culture filtrate of *R. solani*

Table 2. *Effect of oxidized products of chlorogenic acid on* PG *activity of A. oryzae.*

Concentration in (μm/ml)	Enzyme activity (units/ml) Phenol + phenolase	Enzyme activity (units/ml) Original*	Inhibited activity (units/ml)	Percentage inhibition
0.5	16.60		11.9	41.8
1.0	13.80		14.7	51.5
		28.5		
1.5	13.20		15.3	53.7
2.0	15.10		13.4	47.0

* PG activity of the culture filtrate of *A. oryzae*.

Inactivation of pectic enzymes by the oxidized products of polyphenols has been reported widely[3,5,6]. This may be held as a mechanism responsible to invalidate the pathogen attack but, such an assumption would be highly debatable[1,4,7]. Patil and Dimond[6] observed the inactivation of *Verticillium* PG by oxidized chlorogenic and caffeic acids as a complex non-specific process that begins with the 1, 4 addition of the amino groups of the enzyme to quinone molecule. Lyr[5] reported that the inactivation of *Coniophora cerebrella* PG by the oxidized phenols is due to the oxidation of the sulfhydryl (SH) groups of the enzyme. However, Patil and Dimond[6] reported that the inhibition of PG of *Verticillium* by the chlorogenic acid quinones could be prevented by the addition of certain amino acids to the incubation mixture and suggested that the presence of amino groups, in the incubation mixture, which would compete with the amino groups of the enzymes for the 4 position of the quinones reduces the inhibition of PG by the quinone.

Thus the observed inhibition of PG of *R. solani* and *A. oryzae* by the oxidized products of chlorogenic acid could either be due to the oxidation of the SH groups of the enzyme by the quinone or by the formation of 1, 4 addition compounds by the quinone with the amino groups of the enzyme.

However, studies on these aspects would reveal the exact mode of action of the oxidized products of chlorogenic acid on the PG of *R. solani* and *A. oryzae*.

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STUDY OF POLLEN MITOSIS AND DETERMINATION OF GAMETIC CHROMOSOME NUMBER IN OLEACEAE

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STUDY of nuclear division in pollen is interesting and important for the correct determination of the gametic number of plant chromosomes. A convenient and effective technique of staining chromosomes in pollen for observing their division, form and behaviour has not so far been standardised. Light miscroscopic examination of pollen mitosis is usually hindered by the presence of the exine enveloping the inner protoplast. There are several plants like the many members of Oleaceae whose pollen although stainable by dyes do not ordinarily germinate under *in vivo* or *in vitro* conditions[1]. As such, their nuclei or chromosomes in division are unavailable for study at the time of pollen tube formation. This necessitates separation of the inner protoplast from the exine. It is extremely inconvenient and difficult to achieve their separation by mechanical means like pressing or tapping with needles. Further, when the protoplast is taken out by such methods the intine and the inner cell contents are usually broken asunder resulting in the scattering of chromosomes. A more convenient method of separating the exine from its inner protoplast with intact intine at the same time staining the nuclei has been developed[2]. This method has been taken advantage of presently for staining and observing the chromosomes in pollen mitosis and determining the gametic chromosome number of several species of Oleaceae.

The materials for the present investigation were the pollen from several members of Oleaceae collected from different places in South India. Pollen grains from either freshly collected flowers or from those fixed in 1 : 3 acetic ethanol were placed in a drop of LPO (lactopropionic orcein previously prepared by dissolving 2 g Gurr's natural orcein in 100 ml 1:1 lactic acid and propionic acid and diluting it to 45 % with distilled water[3]) on a slide, covered with a cover glass, sealed and kept for 2 hr before observation under the light microscope.

It was observed that the protoplast of the pollen bulged out through the pore and got ejected completely out of the exine without rupture of the membranous intine or any apparent injury or alteration of the inner cell contents. At the same time, the chromosomes at different stages of division such as metaphase (figure 1) or anaphase were intensely stained and distinctly discernible. By this method the gametic chromosome numbers of several members of Oleaceae have been determined. *Jasminum calophyllum* Wall., *J. chinensis* L., *J. pubescens* Willd., *J. primulinum* Hensl., *J. grandiflorum* L., *J. sambac* Wight. (Var. 'gundumalli' double whorled and single whorled) had 13 each, *Ligustrum ovalifolium* L., *L. lucidum* Ait., *L. robustum* Blume., *Linociera courtallensis* Bedd., and *Olea dioica* Roxb. had 23 each and *Nyctanthes arbor-tristis* L. had 22 gametic chromosomes. By comparison with the gametic numbers both somatic and meiotic numbers of different species of Oleaceae could further be confirmed.

The above method of staining chromosomes in pollen is simple, rapid and extremely convenient; at the same time it preserves the structure of the cell without producing artefacts. It enables microscopic observation of gametic chromosomes on a clear cytoplasmic background with sufficient contrast. In addition to ejection of protoplast as observed presently it is further noted that rupturing of cell mem-

Figure 1. Pollen protoplast of *Jasminum pubescens* treated with LPO showing 13 gametic metaphase chromosomes × 3000.