Mycopath (2019) 17(2): 79-88 **Research Article**

Biochemical and molecular analysis of *Alstonia scholaris* leaf galls induced by *Pauropsylla tuberculata* (Psyllidae)

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Abstract

Anatomical and biochemical alterations in foliar galls of *Alstonia scholaris* (L.) R. Br. induced by the insect *Pauropsylla tuberculata* (Psyllidae) are described and quantified in this study. Galls may occur, isolated or agglomerated on both surface of the leaf. The insect along with the egg deposits some physiologic fluid which acted as a stimulant for the induction of the gall. These galls caused changes in morphology and physiology which involved in biochemical changes. There was a decrease in level of chlorophyll in galls which in turn decreased photosynthetic area. Chlorophyll *a* and *b* decreased as galls progressed. Newly developed immature galls showed increase in protein contents up to two folds than healthy tissues. High proline contents were observed in mature galls than immature galls showing stressed condition of galled tissues. As disease progressed, remarkable increase in catalase activity was observed indicating response of plants defence mechanism. Steady increase in phenol contents was observed as disease progressed from immature to mature galls.
Keywords: *Alstonia scholaris*, Disease induction, Galls, Psyllidae.

Introduction

Alstonia is a genus of evergreen trees and shrubs, and belongs to family Apocynaceae. The genus *Alstonia* contains about 40–60 species, native to tropical and subtropical regions of southeast Asia, Africa, Polynesia America, and Australia, with most species in the Malaysian region. In Central America, there is only one species, growing mainly in the form of shrubs, named as *Alstonia longifolia* (Markgraf, 1974; Forster, 1992). Virtually all plants are hosts to bacteria and fungi that can be classified as endophytes. The term endophyte was defined as microbes that colonize living internal tissues of plants without causing any immediate, overt negative effects (Stone *et al.,* 2000). This particular definition implies a mutualistic relationship between the host plant and the endophytic microbe, however the endophytic relationship can range from mutualistic to bordering on pathogenic ass suggested by Petrini (1991).

The most frequently encountered endophytes are fungi. It has been estimated that there may be over a million different fungal species on this Earth, of which only a small fraction (5%) have been identified (Hawksworth and Rossman, 1997). There are also many bacteria that exist as plant endophytes, and indeed in most instances they coexist with endophytic fungi. The existence of endophytes has been known for over one hundred years (Cole and White, 1985). Botanists have carried out much research into the plant/endophyte relationship, especially for grasses such as tall fescue, where it has been shown that endophytes produce toxins that discourage insects and other grazing animals. It wasn't until the past decade or so, however, that endophytes have been studied for their potential as novel sources of effective new drugs (Bultman and Murphy, 2000). Galling habits have evolved convergently among and within numerous insect lineages indicating that the phenomenon is highly adaptive. Galls are formed by manipulation of plant tissues by gall-forming insects to their own benefit (Weis *et al.,* 1988 ; Shorthouse and Rohfritsch, 1992).

Gall-inducing insects whose lifecycles are intimately linked with those of their host plants (Raman, 2003) are the most well studied of these interactions, which present contrasting outcomes: in galls induced by gall-inducing gall midges (Diptera: Cecidomyiidae) fungi are used for nourishment, whereas in galls induced by cynipids (Hymenoptera) the fungi are parasitic on the larva and in those induced by aphids and psyllids (Hemiptera) the fungi are saprophytic living on degenerating gall tissues (Taper *et al.,* 1986, Butin, 1992). Insect interactions with fungal endophytes in grasses within the fungal endophytes belonging to Balansiae (Ascomycota: Clavicipitaceae) that occur in grasses, most of the known information pertains to Neotyphodium (Acremonium, sect. Albanosa). Protection of host plants from insects provides strong

support for this consideration with 40 insect species affected by either the endophytes or the alkaloids they produce (Popay and Rowan, 1994; Clement *et al.,* 1994). Therefore, the present study was designed to elucidate the biochemical and morphological changes taking place in *A. scholaris* leaves at different stages of galls development, in order to determine the plant response to leaf galls induced by *P. tuberculata*.

Material and Methods

Plant sampling

Healthy, young gall containing, mature gall containing and leaves without of *A. scholaris* were collected from infested and uninfected trees growing in Lahore, Punjab. Samples of healthy and gall containing leaves by the *Psyllidae* in different developmental stages were collected at the canopy of different *A. scholaris* individuals. Randomly 10-15 mature gall containing leaves were collected from each plant and sampling was done for two months at 15 days interval. The youngest gall developmental stage was determined based on the smallest diameter, observed as a small spot appeared on the leaf blade. The mature leaves without galls and galls of different developmental stages were taken to the laboratory.

Morphological and anatomical study of gall

Morphology of galls was studied in laboratory by using dissecting microscope. Photographs were taken in laboratory as well as in the field of different stages of development of galls. To study anatomy, galls were cut from backside of leaf using sharp blades and insects were observed at different stages from nymph to mature.

These diseased leaf samples were then classified into different stages as follow: 1. Healthy leaves (T1), 2. Young gall (T2), 3. Mature gall (T3), and 4. Perforated gall (T4)

Preparation of leaf extracts for the biochemical analysis

For each set of experiment 10 g of leaf samples were taken, washed and crushed by using liquid nitrogen in already chilled pestle and mortar until it changes into fine paste. In gall containing leaves, first we cut the galls from backside using sharp blade. Then all the insects were removed by using fine needled very carefully and crushed only gall containing portion for accuracy. Later this sample was extracted using 5 mM tris extraction buffer by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then collected and stored at 4 ^{o}C and used it as it needed (Biswas *et al.,* 2014).

Measurements of chlorophylls a, b and total chlorophyll contents

Healthy and diseased leaf chlorophyll content was estimated by crushing them (Bruuinsma, 1963) in acetone for all stages of *Alastonia* plant. The freshly collected leaves were cut into 0.2 cm parts and crushed in acetone by using already chilled Pestle and mortar. The crud leaf extract (1 mL) was mixed with 4ml of 80% (v/v) acetone and stored at room temperature in darkness. This mixture was then centrifuged at 10,000X for 10 min for preparation of supernatant. The resultant pigmented supernatant was then used for estimation of chlorophyll content. For chlorophyll *a*, absorbance was recorded at 645 nm and for chlorophyll *b*, at 663 nm on spectrophotometer against 80% (v/v) acetone blank. Chlorophyll *a*, *b* and total chlorophyll were calculated according to Arnon (1949).

The chlorophyll concentrations are calculated as follows (use 80% acetone as a blank control).

Chlorophyll *a* (mg g^{-1}) = [12.7 × A663 – 2.69×A645] × V/1000 × W
Chlorophyll *a* (mg g^{-1}) = [22.9 × A645 – 4.86×A663] × V/1000 × W
Chlorophyll *a+b* (mg g^{-1}) = [8.02 × A663 + 20.20 × A645] × V/1000 × W

Estimation of total phenols

Estimation of phenols was done by following (Nurmi *et al.,* 1996) and (Ruuhola and Yang, 2006) method. Leaf samples were extracted in 100 mM tris buffer by using a pestle and mortar. Then centrifuged at 10,000 rpm for 10 minutes at temperature 4 °C. Supernatant was then mixed with freshly prepared reagent at the ratio of 1:10. Four sets of experiments were performed each set with four replicates. In each set, three replicate shows stages of disease, while last one represents the control having distilled water instead of our sample. Regents were freshly prepared by mixing an equal volume of 1% ferric chloride ($FeCl_3$) and 1% potassium ferric cyanide. For the healthy leaf group and other three diseased groups, 5 mL of leaf extract and for control 5 mL distilled water were used. As a result, we added 0.5 mL reagent in each set and performed their colorimetric analysis.

Proline contents estimation

Plant tissue samples (0.5 g) were taken and homogenized in 5 mL of 3% sulphosalysylic acid. Then centrifuged at 1000 rpm and the supernatant was collected for the estimation of proline contents. Afterwards, 2 mL of sample (filterate) + 2 mL acid ninhydrin solution +2mL of glacial acetic acid were taken and boiled for one hour at 100 ^{o}C, brick red colour was developed. Cool the reaction mixture at room temperature and add 4 mL of toluene and mixed well. After that, suck the solution on the upper side by Pipette. A standard curve of proline was prepared by taking 5 to 100 µg mL^{-1} concentrations by taking absorption at 520 nm. The absorbance was calculated with the help of spectrophotometer against toluene as a blank. Free proline content in sample is estimated by referring to a standard curve made from known concentrations of proline (Bates *et al.,* 1973) . Following formula was used to estimate the proline contents in sample solutions,

Proline = Reading × 4 \ 115.5 g sample \ 5

Determination of total content of proline on different stages of *Alastonia* galls development was done by ninhydrin determination of proline method.

Catalase activity

Catalase activity was determined by using the standard baseline protocol as described by Aebi (1994). For this purpose 3% H_2O_2 was used as substrate of catalase. The reaction mixtures were prepared by mixing 8 mL of distilled water, 1 mL of 3% H_2O_2 and 1 mL of enzyme extract. For the control group, mixture was containing 1mL distilled water instead of the enzyme extract was used. This enzymatic reaction was kept only for 20 minutes which was then terminated by adding 5 mL of 10% H_2SO_4 in each set. Titration of each flask was done against N/25 $KMnO_4$ solution, until a persistent pink colour was obtained.

Protein quantification by Bradford assay

The Bradford protein assay was used to measure the concentration of total proteins in the samples. The method is based on the principle of binding the protein molecules to coomassie dye under acidic conditions which resulted in a colour change from brown to blue. This method measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to the formation of the protein-dye complex (Bradford, 1976).

Bradford reagent

Dissolved 500 mg of Coomassie Brilliant Blue G-250 in 50 mL of absolute methanol and 100 mL 85% (w/v) phosphoric acid (H_3PO_4) was added. Then, acid solution mixture was slowly poured into 850 mL of H_2O and let the dye dissolve completely. Then filtered using filter paper to remove the precipitatesandstored in a dark bottle at 4 °C.

Five dilutions of a protein (BSA) standard with a range of 2 to 15 µg protein were prepared. Then unknown protein samples were diluted to obtain protein concentration 2 times. Afterwards, 100 µL each of standard solution and unknown protein sample was mixed to an appropriately labelled test tube. For the standard curve, 100 µL buffer was added instead of the standard solution. Add 5 mL of Bradford reagent to each tube and thoroughly mixed. Incubate at room temperature for at least 10 min. The absorbance was measured at 595 nm wavelengths.

SDS PAGE analysis of leaf galls of *A. scholaris*

To study the protein profile SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed, unidimensional SDS-PAGE apparatus were used (Laemmli, 1970) 12% separating gel and 5% stacking gel were prepared by using their standard recipes. SDS Page was run in the mini vertical system. For this 2.5 µg of protein was loaded in each sample well along with 10 µL sample buffer which contain bromophenol blue as tracking dye. Protein marker was also loaded in one well for comparison of molecular bands for samples. The separating gel was run at a constant voltage of 80 V for one hour while stacking gel was given 120 V until all the dye get removed from sample. Then gel was shifted for staining in coomassie brilliant blue overnight. Relative mobility (Rm) of the protein bands was determined and Zymograms were constructed. Gel was stored in 3% acetic acid and photographed (Deepika, 2012).

Statistical analysis

Data analyses were performed at $P \leq 0.05$ with the help of Statistix 8.1 and Microsoft Excel®. The data were subjected to the ANOVA and means were compared according to the Tuckey's HSD test.

Results

Morphology and identification characters of *Aspergillus flavus* isolated from *Alstonia* leaf galls

Colonies of *Aspergillus flavus* was grown 3.7 cm in diameter within 7 days on MEA plates at 28 °C. Morphologically Mycelium color was white, colony color was dull green and reverse cream colored, exudates absent, and colony texture was spory (Table 1). Conidial heads were dark green in appearance, radiated 25 to 35 µm in diameter. Vesicles were 15-20 µm in diameter, hyaline and pyriform in shape. Conidiophore were roughened, thick walled, 10–12 µm in width and more than 3 mm in length. Sterigmeta were biseriate, phailides 4–5 µm in size. Conidial attachment with condiophore was single. Conidial shape was round to globose in shape, thick walled 3–5 µm in size and smooth surface (Fig. 2).

Chlorophyll a contents

In this study the remarkable deficiency in chlorophyll *a* contents was observed (Fig. 3), where it was clearly showed the huge difference between healthy and different infected stages of *A. scholris* plant. T1 indicates the healthy plant, T2 indicates the first diseased stage or/and A, T3 indicates the 2nd diseased stage or/and B and T4 indicates the 3rd diseased stage or/and C stage of plant. In healthy stage (T1) chlorophyll *a* contents were about 10.8 mg g^{-1}, while in T2, during the first diseased stage, chlorophyll *a* contents are drastically decreased from 10.8 mg g^{-1} to about 1.7 mg g^{-1} continually decreasing chlorophyll contents in 2nd and 3rd diseased stages as 1.5 mg g^{-1} and 0.9 mg g^{-1}, respectively (Table 2).

Chlorophyll b contents

Chlorophyll *b* contents were decreased due to fungal infection in *A. scholris* plant (Fig. 4). At this case (T1) chlorophyll *b* contents were about 7.3 mg

g^{-1} in healthy stage, that was sufficient value for Chlorophyll *b* in *A. scholris* (Bruuinsma, 1963), while during the first diseased stage (T2), chlorophyll *b* contents were drastically decreased from 7.3 mg g^{-1} to about 1 mg g^{-1} continually decreasing chlorophyll contents in 2nd and 3rd diseased stages as 0.9 mg g^{-1} and 0.3 mg g^{-1}, respectively.

The changes in the level of chlorophyll *b* contents in healthy and infected leaves are shown in along with analysis of variance in Table 2.

Total chlorophyll contents

Total chlorophyll contents in *A. scholris* are shown in Fig. 5, where T1 describes the healthy plant contents and T2, T3 and T4 describes the different infected stages of plant. Total chlorophyll contents were found about 17.2 mg g^{-1} in health stage while drastic change was observed during infected treatments. Total chlorophyll contents were 2.5 mg g^{-1}, 2.1 mg g^{-1} and 1.1 mg g^{-1} during T2, T3 and T4, respectively.

Proline contents

Proline contents in *A. scholris* are shown in Fig. 6, where T1 describes the healthy plant contents and T1, T2 and T3 describes the different fungal infected stages of plant. Proline contents in healthy stage (T1) 0.034 µg g^{-1}, while during infected stages it was increased as 0.76 µg g^{-1}, 0.56 µg g^{-1} and 0.47µg g^{-1} in T2, T3 and T4, respectively.

Phenol contents

Phenol contents in *A. scholris* are shown in figure 7, where T1 describes the healthy plant contents and T1, T2 and T3 describes the different fungal infected stages of plant. During the healthy stage (T1), phenol contents are 0.28%, while these are increased in first fungal infected stage (T2) as 0.3%. In 2nd fungal infected stage T3 phenols are 0.35% while these are remarkably decreased in 3rd stage of fungal infection as 0.12%. Data for phenol contents of *A. scholaris* tree and their analysis of variance are presented in (Table 2). Fungal infection in different treatments caused a significant reduction in total chlorophyll contents at ($P \leq 0.05$).

Catalase contents

During the healthy stage (T1), Catalase contents were 0.45 mg while increased in first fungal infected stage (T2) to 0.62 mg. In 2nd infected stage (B) T3 catalase contents is 0.75 mg while these are remarkably increased in 3rd stage of infection as 11.5 mg (Table 2).

Discussion

In the natural environment, plant galls are a result of close association between plants and arthropods, which have both evolved complex biochemical mechanisms to ensure their existence. The initiation and development of galls, mediated by arthropod herbivory, exposes the host plants to high oxidative stress and induces biochemical and physiological alterations that may include the production of oxidative enzymes as a defense against natural enemies(Shorthouse and Rohfritsch, 1992; Oliveira and Isaias, 2010; Oliveira and Magalhães *et al.,* 2010). Gall forming species invade plants and produce harmful regulating chemicals that cause there organization of plant tissue and formation of characteristic structures, galls (Chaman *et al.,* 2001; Formiga *et al.,* 2011). Galls in most cases remain harmless to the host plant but can become a serious problem when they affect the young parts of the plant.

Galls on *A. scholaris* caused by *Pauropsylla tuberculata* is a covering pouch gall in which the plant tissue grows around and above enclosing the cecidozoa that lies initially exposed on the surface of the organ. Many authors have reported different morphological types of galls on the same plant caused by different insects (Arduin*et al.,* 1991;Scareli-Santos and Varanda, 2003). In *A. scholaris* only one type of gall was observed. According to (Shorthouse and Rohfritsch, 1992) young plant tissues are regarded to present responses against inducing insects when compared to already differentiated tissues. Galls in *A. scholaris* occur on both young and mature leaf tissues indicating that tissues in such species react against the inducing insects regardless the leaf age. Similar results have been found in other species (Arduin and Kraus, 2001 ; Scareli-Santos and Varanda, 2003 ; Scareli-Santos *et al.,* 2008).

Eggs of *P. tuberculata* deposited on the leaves triggers the induction of gall. The nymph stages feed on the leaf, where the eggs were deposited and stimulate gall development by translocating a chemical stimulus on the adaxial and abaxial side of the *A. scholaris* leaves. Shrinkage and dying up of cells lining the opening in the mature gall has been observed. The size of the ostiole increases facilitating the escape of the insect nymph. The nymph moves through this cleaved passage towards the exit, moults and escapes as an adult winged insect. At gall senescence, around the insect chamber and the exit channel a healing tissue is formed.

Living organisms face a variety of internal and external stresses to which they must respond in order to maintain ecological equilibrium. The resistance of plants to disease as a result of host-pathogen interaction involves morphological and biochemical changes depending upon the plants' response to infection. *A. scholaris* exhibited remarkable physiological and biochemical changes after psylloid herbivory. Net chlorophyll content in fresh leaves of *Alstonia* was found to be much higher than that of galled leaves. This may be due to a reduction of the chlorophyllous area of infected leaves due to gall formation. Chlorophyll loss is an indicative symptom

of psylloid herbivory. Chlorophyll content of gall tissues showed a decrease as growth progressed. This loss of chlorophyll is responsible for the decolorzation of the area of the leaf where egg was laid in *Ficus* leaves (Moghe, 1980). The low chlorophyll content in galled tissues was due to the loss of palisade tissues, disappearance of chloroplast and modifications of spongy mesophyll.

An increase of proline content in the galled leaves of *A. scholaris* occurs compared to the ungalled leaves. Induction of proline in galled tissues indicates that this has been produced due to the stress. Proline is produced as a defence mechanism to protect from invaders (biotic stress) or stress factors (abiotic stress) and is believed to be an adaptive response to the altered conditions. Increase in proline content was observed in galled leaves of Populus (El-Akkad, 2004). Proline accumulation is known to be a response to stress condition in plants (Gibon *et al.,* 2000).

Secondary metabolites, especially phenolics, are well known for their role in plant defenses against insect herbivory by inducing oxidative responses in plants (Ni *et al.,* 2001). Generally, when a plant is infested, its phenolic content increases as a consequence of a defense reaction to the infestation. Increased phenolic content after psylloid herbivory, which exerted oxidative stress on the host plants, was observed in gall infested leaves as compared to fresh leaves of Alstonia (Isobe*et al.,* 2006). Synthesis of diverse plant proteins are believed to be of importance in defence is also known (Reinbothe *et al.,* 1994). This led to increased antioxidant enzyme activities which serve as a defense system in plants against pathogen attack (Nurmi *et al.,* 1996; Felton *et al.,* 1989; Tscharntke *et al.,* 2001). The increase in catalase activity (Felton and Duffey, 1991) in galled leaves indicates that stress and the release of secondary metabolites is a defensive mechanism against invaders (Hiraga *et al.,* 2001; Ruuhola and Yang, 2006). These enzymes catalyze the conversion of plant diphenols to highly reactive quinines which causes malnutrition in developing insects (Gibon *et al.,* 2000). Defensive proteins that block the action of proteolytic enzymes from herbivores are found in legumes, tomatoes and other plants. These proteins, known as proteinase inhibitors, rapidly seem to accumulate throughout plants that are being fed upon by insects and even accumulate in undamaged areas of plants that are far from the initial feeding site (Ananthakrishnan, 2001). So it can be concluded that when plants are attacked by insects they generate signals and one of these signals is the initiator of expression of certain polypeptides that may be useful in providing the basis for new crop protection strategies.

Increase in total protein contents was observed as disease progressed from healthy to diseased. The higher protein concentration observed in the galled tissue corroborates the observations of (Mehalingam, 1999; Scareli-Santos and Varanda, 2003). The formation of gall requires mechanical and chemical stimuli. The fluid which probably contains enzymes and other cecidogenic substances released/injected into the plant by the insect at the time of egg laying triggers gall induction. The action of the stimulus leads to the formation of new tissues, which cover the nymph in order to isolate and to eliminate the invader, the gall forming insects (Arora and Patni, 2001; El-Akkad, 2004).

In the present study, the electrophoretic banding profile of total soluble proteins of 6 stages of leaf gall of *A. scholaris* exhibited presence versus absence type of polymorphism, reflecting thereby, and differential synthesis of proteins in the gall at different stages (Uritani, 1971). The present investigation on SDS denatured proteins showed differences in number of bands, band width and intensity among different stages of leaf galls of *A. scholaris*. In initial stages of gall the proteins are showing the same banding pattern in the zymogram but in mature stages some extra dark bands are visible, in older stages the number of bands becomes reduced and in last stage only one band is visible (Choi *et al.,* 2004). The present findings confirm the presence of polypeptide bands of heterogeneous molecular weight and varying intensity in *A. scholaris* at different leaf gall stages while undergoing the biotic stress. The unburst galled tissue showed almost two fold increases in the protein content. As the protein content showed an initial increase and registered the highest level during the young galled stage of their development and declined thereafter in the mature burst galled tissue where in the insect had already exited out from the chamber (Albert *et al.,* 2011). Synthesis of diverse plant proteins are believed to be important in defense mechanism (Reinbothe *et al.,* 1994).

The present study has also shown that the galls of *A. scholaris* are not an uncontrolled tumour growth. Their ontogeny follows a well-defined sequence, and produces an equally well defined morphological & anatomical structure. These alterations reveal the plasticity of plant tissues which may be controlled by insects. According to (Taft and Bissing, 1988), presence of the inducer leads to a rupture of the cellular process of the host, and also results in an active redirection of the existing ontogenetic patterns, to the benefit of the inducer. (Schönrogge *et al.,* 2000) detected similar proteins in seeds and in gall tissue suggesting that gall formation might involve the ectopic expressions of genes of other structures of the host plant.

In addition to the explanation that gall inducing insects inhabit a highly specialized habitat, viz., the gall, for nutrition, another opinion is that a gall is an ‘enemy free’ space protecting the inducing insects from predators and parasitoids (Price *et al.,* 1987). Evidence shows that galls inducing insects are susceptible to heavier levels of relatives (Hawkins *et*

al., 1997). A strong selection pressure for achieving a greater level of protection *e.g.* greater level of hardness and thickness of gall walls, prevails on any gall inducing taxon (Stone and Schönrogge, 2003) to reduce the vulnerability of their off-springs to parasitoids and predators.

Conclusion

It can be concluded from the present study that due to interaction between insect and plant tissue certain physiological and biochemical changes occur which lead to hypertrophy and hyperplasia and gall formation takes place. Generation of number of cells requires high amount of protein so the young and mature gall tissue shows high difference in protein concentration as compared to the normal leaf tissue. This study confirms that when plant is attacked by the pathogens, they inject some elicitors and lead to the synthesis of different type of enzymes and some specific proteins at high amount which is a response of plant against the biotic stress to overcome with it. Insects trigger the defence mechanism of the plant which results the gall formation due to initiation of some biochemical reactions and physiological activities.

Acknowledgement

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Table 1: Morphological characterization of *Aspergillus flavus* isolated from *Alstonia scholaris*.

Isolated from	Vesicle shape	Colony Size (cm)	Texture	Colony Colour	Conidia Size (um)	Relative abundance	Vesicle
Alstonia scholaris	Biseriate	4.3	Powdery white	Front (Light green to white) Reverse (olive brown)	2-3	High	Globose at 100 X 25 divisions

Table 2: Chlorophyll a (mg g^{-1}), chlorophyll b (mg g^{-1}), total chlorophyll contents (mg g^{-1}), proline contents (µg g^{-1}), phenolics (%) and andcatalase contents (mg) of *Alstonia scholaris* at different stages of fungal infection.

Treatments	Chlorophyll *a* (mg g^{-1})	Chlorophyll *b* (mgg^{-1})	Total chlorophyll contents (mgg^{-1})	Proline contents (ugg^{-1})	Phenolic contents (%)	Catalase contents (mg)
Healthy (T1)	10.8a	7.3a	17.2a	0.34b	0.28a	0.45b
Stage A (T2)	1.7b	1b	2.5b	0.76a	0.30a	0.62b
Stage B (T3)	1.5b	0.9b	2.1b	0.56a	0.35a	11.5a
Stage C (T4)	0.9b	0.3b	1.1b	0.47a	0.12b	0b

Means in each column with similar letters (a) did not differ significantly at $P \leq 0.05$.

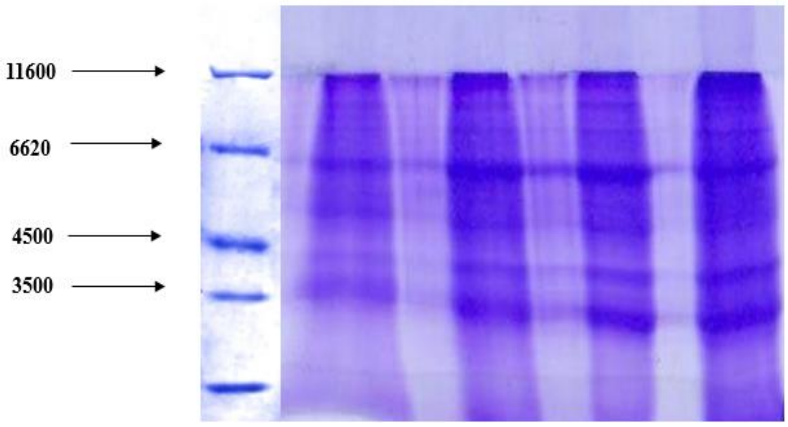

Fig. 1: Total protein content estimation on SDS-PAGE showing the variable protein levels at different stages of leaf gall formation in *A. scholaris*.

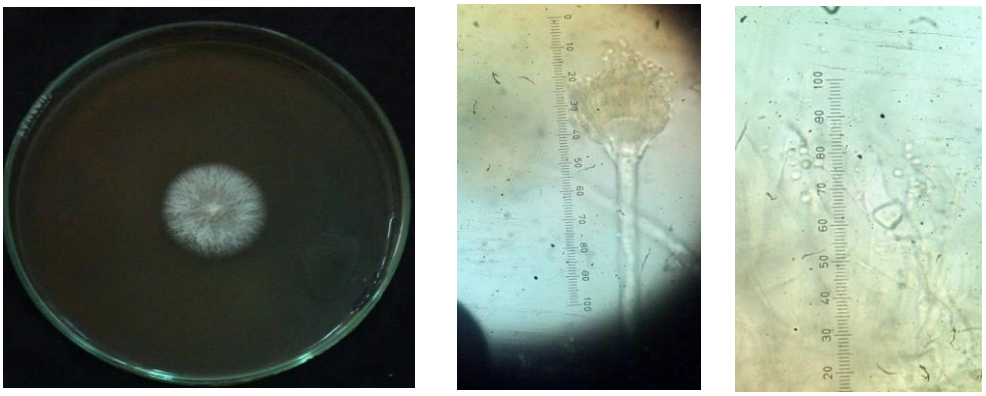

Fig. 2: A)- Morphological characterization of *Aspergillus flavus* in MEA medium; **B)-** Microscopic measurement of fruiting bodies; **C)-** Spore microscopy of *Aspergillus flavus* under compound microscope.

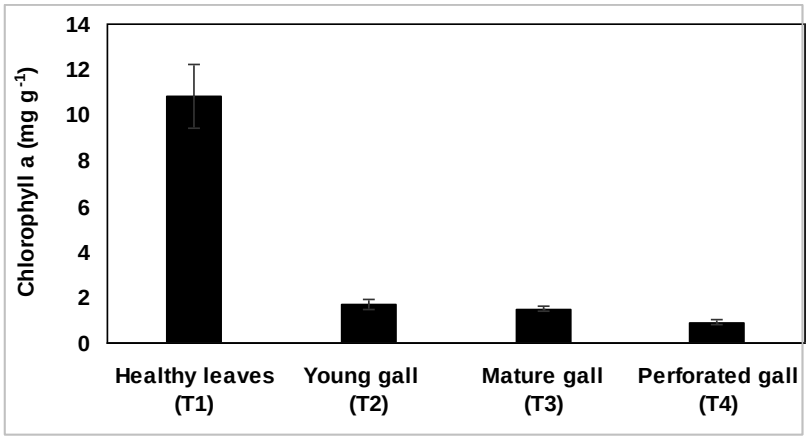

Fig. 3: Estimation of chlorophyll a (mg g^{-1}) contents of *Alstonia scholaris* determined at different stages of gall formation.

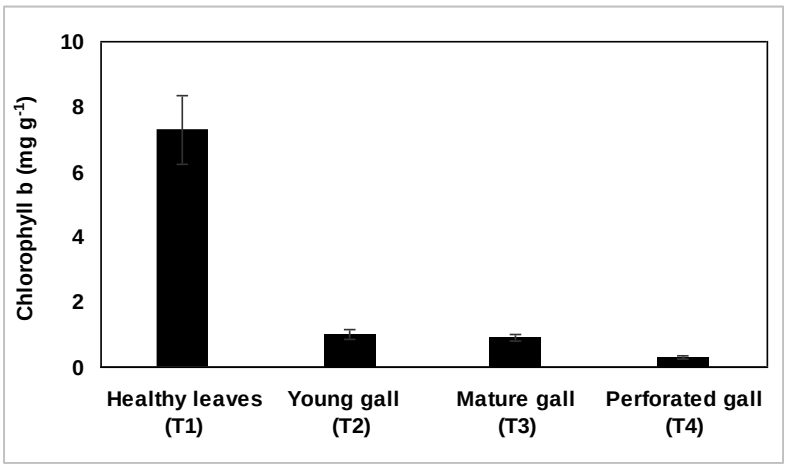

Fig. 4: Estimation of chlorophyll b contents of *Alstonia scholaris* at different stages of disease development.

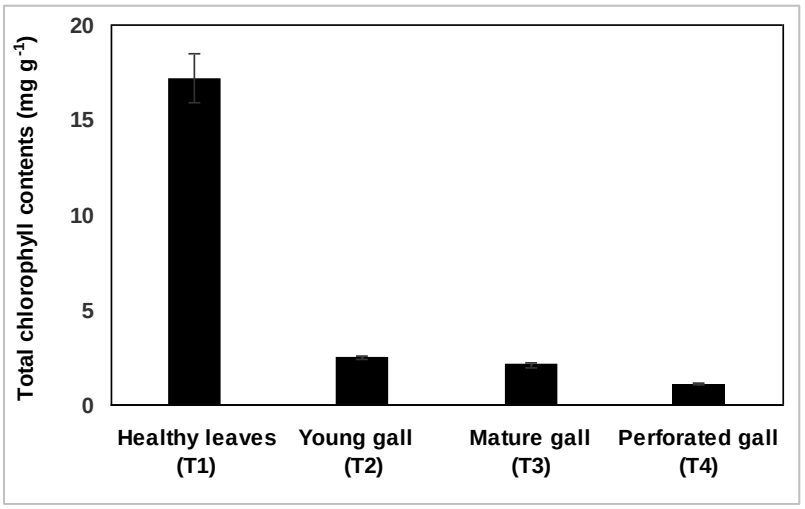

Fig. 5: Estimation of total chlorophyll contents of *Alstonia scholaris* at different stages of fungal infection.

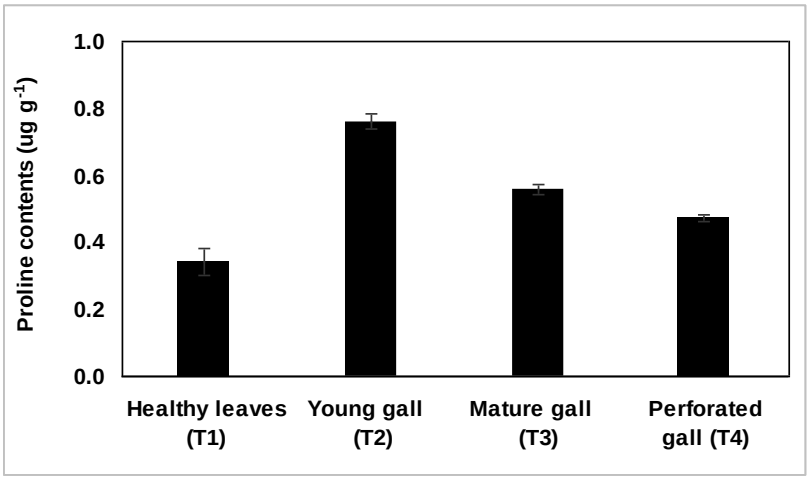

Fig. 6: Total proline contents of *Alstonia scholaris* at different stages of infection.

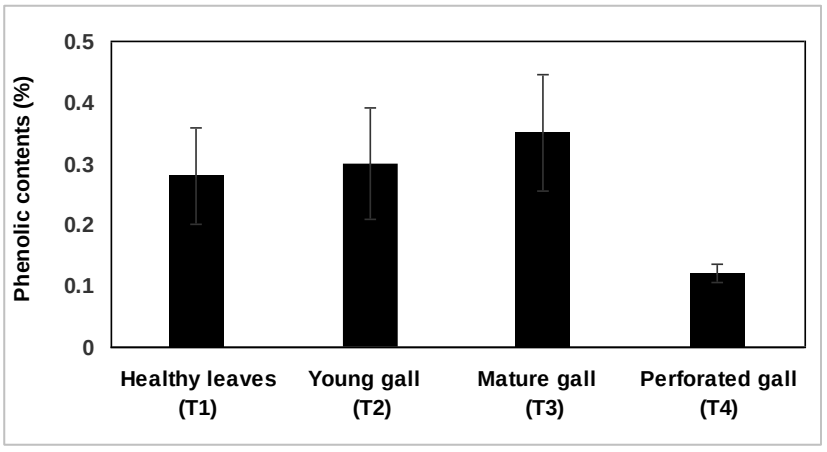

Fig. 7: Effect of different stages of infection on phenolic contents in *Alstonia scholaris*.

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