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Histochemical changes in *Piper nigrum Linn* leaves infected with galls caused by *Diptera*

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ABSTRACT

Piper nigrum Linn. (Black pepper) belongs to the family Piperaceae and an economically and medicinally important spice and is a native of Southern India. The gall tissues have shown various structural and physiological changes in the host tissues. The normal and gall tissue showed differential behaviour in terms of the metabolites and enzymes. The Diptera comprises a large group of insects including the common flies, mosquitoes, gnats and midges are characterised by the position of only the mesothoracic pair of wings. Some of them are predaceous, others feed on nectar or plant sap and decaying animal and vegetable matter and few others are blood sucking. When feeding on plant tissues these insects and mites inject or secrete a chemical substance into the plant that causes the plant to grow abnormally and produce a gall. Stimulus for gall formation is usually provided by the feeding stage of the insect. The remain in the gall as inquilines until the completion and maturity of their life stages and emerges from the exit holes.In present investigation, The intensity of starch, cellulose, carbohydrates, proteins, lipids, lignin, tannins and the enzymes viz. acid phosphatase, peroxidase and polyphenol oxidase was observed in diseased leaf of Piper nigrum and estimated and the results have been discussed in the light of pathogenicity, induced by *Diptera*.

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INTRODUCTION

Piper nigrum Linn. (Black pepper) belongs to the family Piperaceae and an economically and medicinally important spice it is a native of Southern India, especially the malabar coast, growing in rich soil in the shade of trees, to the trunks of which it adheres by means of ivy-like rootlets. Coconut, arecanut, jack fruit, mango, subabul are the other supports used in homestead gardens. From its climbing habit it has received the name of Pepper Vine, and in cultivation the plants are often trained on artificial supports. The flowering and fruiting take place irregularly, the berries taking about five or six months to come to a proper state for gathering, which is before they are fully ripe. They are used as a flavouring spice and also curing ailments like Tonsilitis, intestinal helminthiasis, tooth ache, sore throat, stomache ache, migrane, throat infection, bronchitis, abdominal pain etc.,

Black pepper is, however, principally employed as a condiment, partly for its flavour, and partly on account of its stimulant influence over the stomach, by which it assists digestion.

Gall of *Piper nigrum* induced by unknown Diptera

Plant galls (tumours) are pathologically developed cells, tissues or organs of plants which are formed

mostly by hypertrophy and hyperplasia under the influence of gall inducing agents. The Angiosperms, especially the Dicotyledons are remarkable for bearing a large number of galls. The relative abundance of galls on different parts depends primarily on the plant and the gall maker and it is also influenced by a variety of other environmental factors. The aim of histopathological studies is to understand the adaptational strategies involved Leaf galls of *Piper nigrum* are caused by unknown Diptera, as in Table 1. They are hypophyllous, globose, and hard in texture. They are unilocular, green in colour when young and become grey or brown, when mature. They are rough, solitary and pellet-shaped galls, with about 05-30 mm in diameter. Often as many as a dozen galls can be seen on a single leaf blade. A single layered epidermis made up of rectangular cells surrounds the gall. Gall parenchyma is 16-20 layered. The cells of gall parenchyma are compactly arranged. The larval chamber is oval and lies in the centre of gall. A narrow ostiole is present which widens out on maturation and help in the escape of cecidozoa.

The aim of histopathological studies is to understand the adaptational strategies involved The gall formation is initiated in very young leaf buds. The attack of cecidozoa initiates the gall formation. Around the cecidozoa, there is intense mitotic activity as compared to the cells lying just below the cecidozoa. This abnormal growth around the cecidozoa forms a young gall.

Histochemical localisation of starch, cellulose, total carbohydrates of insoluble polysaccharides, lignins, proteins, lipids, tannins, and the activity of enzymes viz. acid- phosphatase, peroxidase, and polyphenoloxidase in the gall and normal counterparts of leaf marginal roll galls of *Piper nigrum* Linn. induced by unknown Diptera.

Plant galls are extremely complex, both morphologically as well as physiologically. Infection of any kind brings about changes in the morphology and physiology of the host. Many changes take place at the infection site in plant during establishment of host-parasite relationships. Some of the changes alter the normal metabolism favouring the abnormal growth. It is therefore necessary to understand these changes in the diseased tissue. Qualitative histochemical studies on localization of various metabolites provide an insight into he phenomenon at the cellular level.

The aim of histochemistry is to study the localization and identification of substances and enzyme activities within cells and tissues. Several workers have studied the histochemistry of various plants (Jensen, 1962; Johansen, 1940).

The histochemical studies of insect and mite induced galls have been carried out by many workers (Raman and Gopinathan, 1986). He have reported accumulation of lipids in nutritive tissue of midge gall of *Ficus* spp. Presence of high amount of protein in nutritive tissue of insect gall has been reported by several workers (Gopinathan, 1987; Scareli-Santos and Varanda, 2003). (Motta *et al.*, 2005) have found an altered physiology of the host due to cecidogenesis.

Change in normal metabolism of host following infection is a wide spread phenomenon in a plant. Any symptom due to infection, brought by any pathological organism in plants is always associated with some biochemical changes in the tissues.

The pathogen induces cell response by releasing its secretion, which comprises of auxin, enzymes and toxins to alter the host cell metabolism.

In the present investigation, localization of various metabolites and enzymes in the leaf galls of *Piper nigrum Linn and* their normal counterparts have been carried out. The various metabolites localised were starch, cellulose, total carbohydrates of insoluble polysaccharides, proteins, lipids, lignin, tannins, and the activity of enzymes viz. acid- phosphatase, peroxidase, and polyphenoloxidase.

MATERIALS AND METHODS

Plant materials Healthy and diseased leaves of pepper were collected for the estimation.

Starch

Starch was localized by Iodine-potassium iodide (IKI) reaction method (Johansen, 1940). 2.0 g of potassium iodide was dissolved in 100 ml of distilled water and then 0.2 g of iodine was dissolved in it. Fresh hand cut sections were placed in IKI solution for few minutes and then mounted in the same solution and observed.

Results are presented in Table 2. Starch, the most important, carbohydrate reserve in plants is localized as black granules in cells. In leaf gall caused by Diptera in *Piper nigrum* Linn, moderate starch content was observed in gall parenchyma and high intensity was observed in epidermis.

Cellulose

IKI-H_2SO_4 method of (Johansen, 1940) was followed for localization of cellulose.

Fresh hand cut sections were kept in IKI solution for 15 minutes and mounted on a glass slide. Through the sides of the cover slip a drop of 65% sulphuric

Table 1: Plant galls studied in present investigation

S. No	Host Plant	Organ Affected	Gall incitant	Place of Collection
1	*Piper nigrum Linn*	Leaf	Unknown Diptera	Thiruvanthapuram, Idukki, Ernakulam, Quilon, Kottayam

Table 2: Histochemical localization of metabolites and enzymes in different regions leaf gall and normal leaf of *Piper nigrum Linn.* induced by unknown *Diptera*

Metabolites	Normal or Gall	Epidermi	Palisade	Spongy Parenchyma	Vascular Bundle	Gall Parenchyma
Starch	N	+	+	+	++	x
	G	+++	X	X	x	++
Cellulose	N	++	-	++	++	x
	G	+++	X	x	x	++++
Carbohydrates	N	+++	++++	++	++	x
	G	+++	X	x	x	++++
Protein	N	+	++++	+	+	x
	G	++	X	X	x	+++
Lipids	N	++	++	+	+	x
	G	+++	X	X	x	+++
Lignin	N	-	-	-	+++	x
	G	-	X	X	x	+++
Tannins	N	++	+	+	+	X
	G	++	X	X	x	+++
Acid phosphatase	N	-	+++	++	-	X
	G	+	X	X	x	+++
Peroxidase	N	++	+++	+	+	X
	G	+++	X	X	x	+++
Polyphenol oxidase	N	+++	++++	++++	+++	X
	G	++	X	x	x	+++

N = Normal
G = Gall
- = Nil
+ = Low intensity
++ = Moderateintensity
+++ = High intensity
++++ = Very highintensity
x = Tissueabsent

acid was allowed to diffuse in and the sections were immediately observed.

Results are presented in Table 2. In gall of *Piper nigrum* induced by Diptera, cellulose was observed to be high as compared to normal. It was localized intensely in epidermis followed by parenchyma whereas in the normal counterpart it was observed in moderate intensity.

Total carbohydrates of insoluble polysaccharides

Periodic Acid Schiffs (PAS) reaction (Hotchkiss, 1948; McManus, 1948) was followed for localization of total carbohydrates of insoluble polysaccharides. Standard Schiff reagent was prepared by dissolving 0.5 g of basic fuchsin and 0.5 g of sodium metabisulphite in 100 ml of 0.15 N HCl. This mixture was shaken at an interval of 2-3 hrs. until the dye was converted to fuchsin sulphurous acid.

This solution was passed through fresh activated charcoal and the process was repeated till a clear filtrate was obtained. Fresh hand cut sections were kept in 0.5% periodic acid solution for 15-30 minutes. After washing in running tap water for 10 minutes these were treated with Schiff's reagent for 15 minutes. The sections were washed in distilled

water and then placed in 2.0% sodium bisulphite solution for 1-2 minutes. After rinsing in running water for 5-10 minutes, sections were mounted in glycerine and photomicrographed.

Results are presented in Table 2. In gall caused by Diptera in *Piper nigrum,* carbohydrates were localized in very high intensity in epidermis and parenchyma although the intensity was moderate in the normal counterpart.

Proteins

Localization of proteins was done by Amido-black method (Von and Wieme, 1959). Amido-black dye was prepared by adding 0.5 g of amido-black solution containing 5.0 g of mercuric chloride and 5.0 ml of glacial acetic acid in 100 ml of distilled water. The stain was filtered and used.

Fresh hand cut sections were stained by amido-black dye for 2-3 minutes and subsequently washed in 2.0% acetic acid for 5 minutes and later in distilled water. The sections were mounted in glycerine and observed.

Results are presented in Table 2. In *Piper nigrum* induced by Diptera, Protein was localized in high intensity in the gall epidermis and parenchyma while it was present in relatively low intensity in the normal.

Lipids

Total lipids were localized by the method given by (Chiffelle and Putt, 1951).

0.7 g of sudan III dye was dissolved in 100 ml of ethylene glycol. The solution was heated to 100-110^{0}C and stirred thoroughly. The dye was filtered through Whatman No. 2 filter paper and stored in a brown bottle.

Fresh hand cut sections were placed in ethylene glycol for 3-5 minutes and subjected to occasional shaking. The sections were then transferred to the sudan-III dye and stained for 5-7 minutes. These sections were once again transferred to ethylene glycol and distilled water and shaken for 2-3 minutes. After a thorough washing for 3-5 minutes in distilled water, the sections were mounted in glycerine and observed.

Results are presented in Table 2. In leaf gall of *Piper nigrum*, induced by Diptera, the intensity of lipids was found to be high in parenchyma and epidermis whereas it was low in the normal counterpart.

Lignin

Lignin was localized by phloroglucinol HCl test (Johansen, 1940). 1.0 g of phloroglucinol was dissolved in 100 ml of 95% ethanol.

Fresh hand cut sections were placed in phloroglucinol solution. A drop of 20% HCl was added to stain. Sections were removed, mounted in glycerine, observed.

Results are presented in Table 2. In leaf gall of *Piper nigrum* induced by Diptera, high amount of lignin was observed in parenchyma than in the normal counterpart. It was localized intensely in the mechanical tissue of leaf gall and vascular region of normal leaf.

Tannins

Tannins were localized by Lugol's iodine method (Haridass *et al.*, 1985). Lugol's iodine solution was prepared by IKI solution (4 g iodine + 6 g potassium iodide) in 100 ml of water.

Fresh hand cut sections were treated in Lugol's iodine solution for few minutes. To this, a drop of dilute ammonium hydroxide solution was added. The sections were mounted in glycerine and immediately observed.

Results are presented in Table 2. In the parenchyma of leaf gall of *Piper nigrum*, tannins were localized in high intensity while very low intensity was observed in the normal part.

Acid Phosphatase

Acid phosphatase activity was localized by the procedure of (Gomori, 1952) with slight modification.

Substrate solution was prepared by dissolving 0.5 g of lead nitrate in 500 ml of 0.5 M acetate buffer (pH 5.5). To this solution 50 ml of 0.1 M sodium glycerophosphate was added and then its pH was adjusted to 5.0

Fresh hand cut sections were placed in the freshly prepared substrate solution for 2 hours at 37^{0}C. Subsequently the sections were rinsed in distilled water and placed in dilute ammonium sulphide solution for 5 minutes. Sections were once again rinsed and mounted in distilled water, observed.

Results are presented in Table 2. In leaf gall of *Piper nigrum* induced by Diptera, localization of activity of acid phosphatase was observed to be intense in parenchyma and nutritive tissue whereas it was low in normal counterpart.

Peroxidase activity

Activity of peroxidase was localized by the method of (Isaac and Winch, 1947).

Incubating medium was prepared by adding equal volume of 0.1 M phosphate buffer (pH 5.5), 0.1 M H_2O_2 and 0.01 M benzidine.

Fresh hand cut sections were placed in the incubating medium for one hour at 37^{0}C and observed. The

preparation was temporary hence observed.

Results are presented in Table 2. Peroxidase stained brown in colour. Peroxidase activity was observed to be more

The intensity of peroxidase activity was observed to be high in gall parenchyma and gall epidermis. It was found to be slightly less in the cells near the gall cavity while the activity of enzyme was found to be low in the normal leaf.

Polyphenol oxidase

Polyphenol oxidase activity was observed by the method of (Sexton and Hall, 1978).

Incubating medium was prepared by dissolving 50.0 mg of DL 1,3,4-dihydrophenylalanine (DL-DOPA) in 10 ml of 67 mM Sorenson's phosphate buffer (pH 7.0). Fresh hand cut sections were incubated in freshly prepared incubating medium for one hour at 37^0C.

Results are presented in Table 2. The enzyme activity was very high in the leaf gall induced by Diptera. The activity of polyphenol oxidase was observed to be high in the palisade cells of normal leaf.

RESULTS AND DISCUSSION

A change in the distribution of metabolites and enzymes in gall was observed as compared to normal counterparts in all the plants analyzed viz., *Piper nigrum* Linn

The intensity of starch, cellulose, carbohydrates, proteins, lipids, lignin, tannins and the enzymes viz. acid phosphatase, peroxidase and polyphenol oxidase was observed to be high in galled parts as compared to their normal counterparts.

The differential localization of metabolites and enzymes could be attributed to the activity of cecidozoa. A change in the distribution of metabolites and enzymes in gall and normal counterparts was observed in the plant analyzed viz., *Piper nigrum* Linn. The intensity of starch, cellulose, carbohydrates, proteins, lipids, lignin, tannins and the enzymes viz. acid phosphatase, peroxidase and polyphenol oxidase was observed to be high in galled parts as compared to their normal counterparts. Increase in lignin in gall tissues has been attributed to hyperactivity of peroxidase and polyphenol oxidase. Increase in amount of tannins in gall tissues could be attributed to the higher incidence of activities of polyphenol oxidase and peroxidase. A higher metabolic activity in cells, in the vicinity of feeding area. Higher activity of enzymes in gall tissues has been observed in several insect induced galls (Gopinathan, 1987).

The microscopic histochemistry of localization of metabolites and enzymes activities within the cells of normal and gall tissues of leaf of *Piper nigrum* Linn conducted to study the changes in starch, cellulose, total carbohydrates of insoluble polysaccharides, proteins, lipids, lignin, tannins and activities of enzyme viz. peroxidases, polyphenol oxidase and acid phosphatases**.** A change in the distribution of metabolites and enzymes in gall and normal counterparts was observed in all the plant parts analyzed. The intensity of starch, cellulose, carbohydrates, proteins, lipids, lignin, tannins and the enzymes viz. acid phosphatase, peroxidase and polyphenol oxidase was observed to be high in galled parts as compared to their normal counterparts.

CONCLUSIONS

The result obtained during the histochemical study showed that the incitant which undoubtedly acted as a trigger agent, left disturbing forces inside the tissue and influenced its anatomy and physiology. In order to acheive the functional elaboration in the cells closer to the feeding cites supported by high incidence of proteins, starch, lipids and higher activity of enzymes appeared very impressive.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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