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Histochemical Analysis of Seed Infected With *Fusarium Oxysporum* and *Rhizoctonia* Solani

Singh Anita[1] and Verma Rashmi[2]
Department of Botany, School of Basic & Applied Science,
Career Point University, Kota Rajasthan, India.
Department of Botany,Govt.College kota(Rajasthan)[2]

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I. Introduction

Lentil (*Lens culinaris* Medik.) is an important cool season legume, grown in more than 70 countries around the world. Canada is the largest lentil producer, contributing nearly 41% of the global production (FAO, 2017). Lentil seeds are a rich source of proteins, carbohydrates, vitamins, minerals, fibers and antioxidants (Roy et al., 2010).

Disease such as Ascochyta blight is caused by *Ascochyta Lentis* Bond & vassil and wilt is caused by *Fusarium oxysporum* f. Sp. Lentis play a major role in reducing lentil yield[4]. Wilt disease appears in the field in patches at both seedling and adult stages. Seedling wilt is characterized by sudden drooping followed by yellowing and drying of leaves and the whole seedling and apparently healthy roots with reduced proliferation.

Present study was undertaken to understand histochemical analysis of seed infected with *Fusarium oxysporum* and *Rhizoctonia solani*.

Key words: histochemical staining, *F.oxysporum, Rhizoctonia solani , Lens culinaris medic.*

II. Materials and Methods:

Histochemical methods were used to study the localization of various metabolites and food reserves in seeds. Seeds carrying natural infection of, *F.oxysporum, Rhizoctonia solani* and healthy seeds were used (Table-1, 2) to analysis proteins,starch,cellulose,phenols and tannins. Methods employed for each are dealt separately.

Total proteins: Total protein was localized by the mercuric bromophenol blue method (Mazia,Brewer and Alfert, 1953; Ruthmann, 1970;Chapman, 1975).

Preparation of stain: 10 gm of mercuric chloride was dissolved in 100ml of 95% ethanol. To this 100 mg bromophenol blue was added.

Staining procedure: Fresh hand cut sections were stained in mercuric bromophenol blue for 15 min in 0.5% acetic acid to remove the excess dye.

The sections were washed in water for 15 min and mounted in glycerin.

Protein stains blue.

Starch: Starch was localized by iodine (IKI) method of Johansen (1940).

Preparation of stain: 2gm potassium iodide was dissolved in 100ml distilled water and then 0.2 gm iodine was added to it.

Staining Procedure: Fresh hand cut sections were placed in iodine potassium iodine solution for a few minutes and then mounted in the

Same solution and observed.

Starch grains appear blue to black in colour.

Total phenols: Phenol was localized by nitroso reaction (Reeve, 1951).

Preparation of stain: Mixture of following reagent was used for staining. (i) 10% sodium nitrite (ii) 10% to 20% urea (iii) 10% acetic acid.

Staining Procedure: Fresh hand cut sections were placed in stains for 3-4 min. and then added 2N sodium hydroxide solution. The section was mounted in glycerine.

Phenols give cherry red colour.

Tannins: Tannins were localized by Lugol's Iodine Method (Haridass and Kumar, 1985).

Preparation of Lugol's Iodine Solution: 4.0gm iodine and 6.0gm potassium iodide were dissolved in 100ml distilled water.

Staining Procedure: Fresh hand cut sections were treated in Lugol's iodine solution for a few minutes. To this a drop of dilute NH_4OH solution was added. The sections were mounted in glycerine.
Tannins appear brown in colour.
Cellulose: Potassium iodide-iodine-sulphuric acid method as described by Johansen (1940) and Purvis et al. (1964) was followed.
Preparation of stain: The I-KI solution was prepared and H_2SO_4 added later.
Staining Procedure: Fresh hand cut sections were stained in iodine potassium iodide solution for 15 min and mounted in the same solution. 65% sulphuric acid was then added through the sides of the cover slipes.
Cellulose cell wall swells and takes a bright blue color.

III. Result

HISTOCHEMICAL STUDIES OF *F.oxysporum* AND *R.solani* INFECTED SEEDS

Total Protein

Normal seed (Fig.-1A)

The localization of proteins was maximum in outer layers of the cotyledon and embryonal axis. The sections of normal seed showed that the cells of cotyledon and embryonal axis contained numerous protein bodies arranged densely and invariable stained blue with bromophenol blue. The cells showed a uniform distribution of stain. The vascular strands of cotyledons took a little weaker stain than other cells. Palisade, hourglass and parenchyma layers of seed coat and hilar region remained unstained showing absence of protein bodies in these cells. Seed tissues rich in protein showed high colour intensity.

Infected seeds (*F. oxysporum)* (Fig. -1B)

The cells of cotyledons and embryonal axis showed discrete light and dark coloured patches of blue colour. The light stained cells of cotyledons and embryonal axis were deficient in protein bodies and appeared vacuolated. The cells were infected with mycelium of *F.oxysporum.* The cotyledonary cells towards seed coat stained little darker than other cells of cotyledons. Different layers of seed coat remained unstained. In general, the intensity of blue colour was than the healthy seeds.

Infected Seed (*R.solani*) (Fig.-1B)

No protein reaction was observed in the layer of seed coat. The cells of cotyledon towards distal and proximal end of seed and embryonal axis were heavily infested by the *R.solani* and showed a negative reaction with stain. The cells were either partly or completely devoid of protein.The intensity of colour in *R.solani* infested seed was very weak than the healthy seeds, indicating high loss of protein due to *R.solani* infection.

Starch

Normal seed (Fig.-1C)

A positive result was observed by IKI reaction reveling the presence of starch in the cotyledons. Starch grains showed their maximum localization in the cells of cotyledons 4-12 starch grains per cell were compactly arranged and distributed uniformly. Seed coat (palisade, hourglass and parenchyma layer), hilum region and vascular strands of the cotyledons revealed absence of starch grains.

Infected Seed (*F. oxysporum*) (Fig.-1D)

The IKI reaction gave a positive result and showed the presence of starch in cotyledons. At places strarch grains appeared small and loosely arranged. The number of starch grains varied from 2-6 per cell, was much lesser than the cells of healthy seeds. The cells which were heavily colonized by the fungus gave a very faint to negative reaction with the stain.

Infected Seed (*R.solani*) (Fig.-1D)

R.solani infested seeds showed similar localization of starch where the cells were completely devoid of starch grains as compared to healthy seeds.

Cellulose

Normal Seed (Fig.-2A)

The IKI-H_2SO_4 reaction gave positive response. The swollen cell walls of the cells of cotyledon and embryonal axis showed bright blue colour. Other parts of the seed showed a negative reaction.

Infected Seed (*F.oxysporum)* (Fig.-2B)

The cell walls of cotyledon of infected seeds with *F.oxysporum* gave a bright blue colouration similar to that of healthy seeds, showing that pathogen has no effect on cellulose.

Infected Seed (*R.solani*) (Fig.-2B)

The cell wall of cotyledon of infested seeds showed the localization of cellulose in the form of faded blue colour. In the infested region the cell walls appeared disrupted which might be due to feeding of *R.solani.*

Total Phenols

Normal Seed (Fig. -2C)

The nitroso gave positive stain reaction for phenolics, which was characterized by cherry red colour in the tissues. The cherry red colour was seen in the different layers of seed coat, funiculus and counter palisade region of hilum. The hourglass and parenchyma cell layers stained little weaker than palisade cells, indicating maximum localization of phenols in palisade cells. Cotyledon and embryonal axis showed negative reactions.

Infected Seed (*F.oxysporum*) (Fig.-2D)

The tissues of infected seed showed maximum intensity of cherry red colour because of increased amount of total phenols due to infection. The palisade and hourglass cells stained little darker than seed coat parenchyma. The cell of cotyledon and embryonal axis also showed weak staining revealing the presence of phenol in these components.

Infected Seed (*R.solani*) (Fig. -2D)

Not much difference in phenol was observed in *R.solani* infested seeds as compared to healthy ones.

Tannins

Normal Seed (Fig.-2E)

Staining with Lugol's iodine solution indicated the presence of tannins (brown colour) in seed coat and outer few layers of cotyledons.

Infected Seed (*F. oxysporum)* (Fig.-2F)

The intensity of brown colour was higher in cell layers of seed coat infected with *F.oxysporum* as compared to healthy seed, indicating an increase in tannins. The formation of brown colour was more towards hilar region. The cells of cotyledon were weakly stained showing its low level as compared to seed coat.

Infected Seed *(R.solani)* (Fig.-2F)

Seeds infested with *R.solani* were similar to that of healthy seeds for tannin.

Discussion

An attempt was made to study the changes in important primary metabolites of lentil seed naturally infected with *F.oxysporum* and *Rhizoctonia solani.*

Proteins

A weak reaction of protein was shown by cells infected with F. oxysporum revealing a decrease in total protein compared to healthy seeds. However, the seeds infested with *C.chinensis* showed negative reaction for protein. The loss in protein was much higher due to *R.solani* as compared with *F.oxysporum* as evident from negative stain reaction of *R.solani*.

Maheshwari, Chaturvedi and Yadav (1984) observed stronger protein reaction in Protomyces macrosporus infected cells of Coriandrum sativum. But, less densely stained proteinoplasts in groundnut leaves infected by Cercospora arachidicola was observed by Vijaya Kumar (1990).

Unlike present studies Ibraheem et.al. (1987) observed an increase in protein content in soybean seeds when inoculated with

cultural filtrates of *Alternaria alternata, Ulocladium spp*., *F.oxysporum* and *F. solani*. Biochemical estimations of soybean seeds infected by *F.oxysporum* and *R.Bataticola* conducted by sharma (1992) and Mathur (1992) showed a continuous decline in protein content.

Decrease in protein contents in pigeon pea seeds infected by different Aspergilli was reported by Shukla et al.(1988). These observations suggests that the level of protein content may vary with host-parasite interaction.

Maheshwari et al. (1984) observed stronger protein reduction in *Protomyces macrosporus* infected cells of *Coriandrum sativum*. But, less densely stained proteinoplasts in groundnut leaves infected by *Cercospora arachidicola* was observed by Vijaya Kumar (1990)

Shukla et al. (1988) found decrease in protein contents in arhar seeds infected by *Aspergilli.* These observation suggests that the level of protein content may vary with host-parasite interaction.

Das and Mitra (1998) found that little leaf infected brinjal leaf tissue contained less amount of soluble protein than in healthy ones.

Starch

Cell in the infected seeds with *F.oxysporum* and *C.chinensis* showed weak reaction for starch as compared with healthy ones. The number of starch grains was also reduced.

Vijaya Kumar (1990) observed decrease in number and size of starch grains in *C.Arachidicola* infected groundnut leaves.

Starch is intracellular and occur as membrane bound granules in cell cytoplasm of cotyledons. However, in diseased seeds, starch was comparatively less, cell arrangement distorted and granules deformed. Similar obsevatices was also observed by Santra (1983). He found that the polysaccharides are less in the infected plants parts and their surrounding as compared to healthy plants of potato. This may be attributed to degradation of polysaccharides by extra-cellular fungal enzymes and/or absorption of the metabolites by the fungus (Hahn et al.1980). Maheshwari et al. (1985) observed high intensity of carbohydrates in the hypertrophied inflorescence axis in *Brassica juncea* caused by *albugo candida*. Infection by *Curvularia lunata, Fusarium moniliforme* and *Phoma sorghina* in sorghum seeds caused significant reduction in size of starch granules as compared with those in healthy seeds.

Cellulose

The presence of cellulose was demonstrated in cell walls of cotyledons and embryonal axis of both the infected and uninfected seeds. The cells of infected seeds showed slightly less amount of cellulose. Similar results also observed by Sharma (1999) in mungbean.

Phenols and Tannins

Parasitic interaction of *F.oxysporum* and *R.solani* resulted increase in phenol content. Similarly tannin was also high in *F.oxysporum* and *R.solani* infected seeds. Many authors have also reported high amount of phenol and tannin in seeds infected with fungus. Both the phenol and tannin are regarded as part of host defense mechanism (Bhatia et al., 1972; Chopra et al., 1974; Farkas and Kiraly, 1962).

Kamble and Gangawane (1987) reported that the total phenol contents of ground nut seeds was increased due to infection of *Curvularia lunata, Aspergillus flavus, PeniclliumFuniculosum, P. various Fusarium oxysporum.* Khirbat and Jalali (2003) observed that the levels of total phenol and tannins contents increased after inoculation of *Ascochyta rabiei* on chickpea. However, increase was significantly higher in susceptible cultivar after 10 days of inoculation as compared to resistant cultivar.

Bhargava, Sharma and Dashaora (2007) observed that resistant cultivar of resistant cultivar of cowpea possed higher concentration of phenols as compared to susceptible, after infection with *Meloidogine incognita.*

The histochemical studies clearly indicate that natural infection of *F.oxysporum* and *R.solani* caused a decrease in total protein and starch, increase in total phenols and tannins while no change in cellulose.

IV. Conclusion

Total proteins

A considerable loss in protein content was observed. The protein bodies in cells of cotyledons were disintegration as the cells appeared vacuolated and gave a weak reaction with mercuric bromo phenol in comparison to the healthy seeds that showed prominent protein bodies giving a dark blue colour.

No difference in proteins content of seeds infested with *R.solani* was observed.

Starch

The number of starch grains per cell as compared to the cells of healthy seeds was much lesser in both the cases.

Cellulose

The cell walls of seeds infected with *F. oxysporum* and that of healthy seeds gave a similar bright blue colouration showing no effect of pathogen on cellulose.

No difference in cellulose content of seeds infested with *R.solani* was observed.

Total phenol

The tissues of seed infected with *F.*oxysporum showed deep cherry red colour in seed coat because of increased amount of total phenols and weak staining in the cells of cotyledons and embryonal axis.

No difference in phenol content of seeds infested with *R.solani* was observed.

Tannins

The intensity of brown colour was higher in cells of seed coat infected with *F. oxysporum* as compared to healthy seeds, indicating an increase in Tannins.Whereas weakly stained cells of cotyledon showed its low level.

No difference in tannin content of seeds infested with *R.solani* was observed.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLE- 1 : DISTRICTWISE NUMBER OF SEED SAMPLES OF LENTIL COLLECTED AND TESTED BY (SBM AND PDA)

District	No. of Samples collected	No. of Samples Studied	
		SBM	PDA
Ajmer	7	7	3
Bundi	10	10	3
Barmer	3	3	-
Bharatpur	15	15	8
Bikaner	12	12	3
Churu	10	10	-
Dausa	10	10	-

Dholpur	30	30	12
Hanumangarh	2	2	-
Jaipur	8	8	1
Jalore	5	5	2
Jhunjhunu	2	2	-
Karauli	10	10	5
Kota	15	15	6
Nagaur	5	5	-
Pali	2	2	1
Sirohi	2	2	1
Tonk	2	2	1
Total	**150**	**150**	**46**

TABLE - 2 DETAILS OF SEED SAMPLES (AC.NOS.) USED FOR COMPONENT PLATING, CLEARED WHOLEMOUNT PREPARATION, MICROTOME SECTION, DISEASE TRANSMISSION USING NATURALLY INFECTED (NI) SEEDS AND THEIR CONTROL.

Pathogens	Histopathology	Disease Transmission	Control		
		NI	Physical	Biological	Leaf Extract
Fusarium oxysporum	3527, 3529	3527, 3529	3527, 3529	3527, 3529	3527, 352
Rhizoctonia solani	3530, 3538	3530, 3538	3530, 3538	3530, 3538	3530, 3538

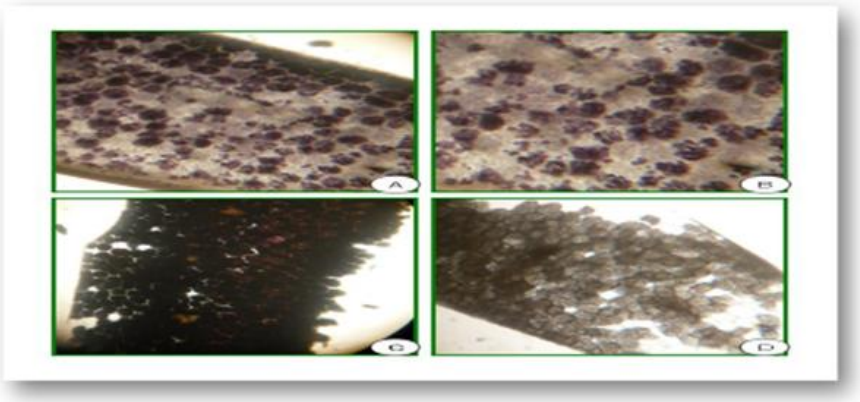

Figure-1

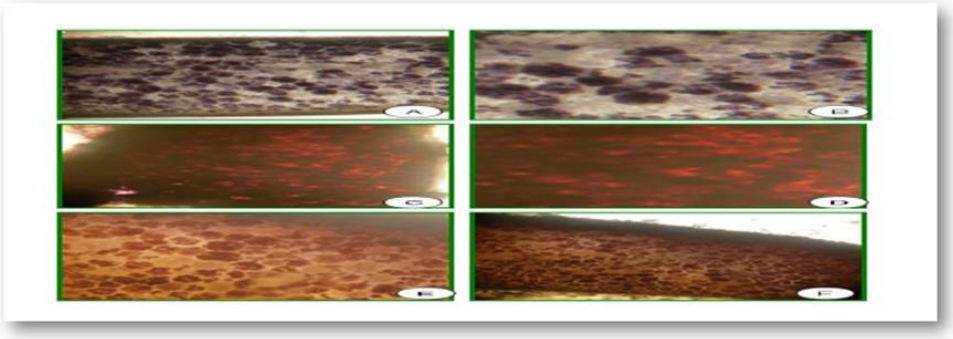

Figure-2

Singh Anita, et. al. "Histochemical Analysis of Seed Infected With Fusarium Oxysporum and Rhizoctonia Solani." *IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT),* 14(12), (2020): pp 37-42.