Histo - chemical mechanism of defense in barley roots attacked by *Fusarium graminearum*

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**ABSTRACT:** Comparative microscopy of inoculated and non-inoculated roots of resistant and susceptible barley genotypes revealed cell wall appositions (CWAs) in rhizodermal cells and accumulation of hydrogen peroxide (H$_2$O$_2$) in cortical cells as defense-related components against *Fusarium graminearum* (Fg), the incitant of common root rot. Resistant genotype Chevron exhibited significantly higher quantities of these defense components than the susceptible genotype Uschi. Temporal and spatial manifestation of CWAs as well as accumulation of H$_2$O$_2$ were directly related with lesser colonization of Fg hyphae in root tissues of Chevron as compared to Uschi. Data based on bright field microscopy suggested that CWAs in rhizoderm and H$_2$O$_2$ accumulation in cortical cells are involved as components contributing for resistance that is characterized as a slow rate of mycelial growth after successful penetration. During the course of present investigations, we pioneered the procedures of root microscopy for studying histo-chemical response of barley roots to infection of Fg. These procedures may be further employed to discern the mechanism of host resistance against a wide variety of *Fusarium* species infecting roots of several agricultural and horticultural crops.

**Key words:** Barley, *F. graminearum*, common root rot, resistance, cell wall appositions (CWAs), Hydrogen peroxide

Root rot caused by *Fusarium* spp. is a common disease of barley in the mediterranean area of the middle east and the pacific northwest of the United States (Mathre, 1982). The pathogen also prevails in warmer areas of south Asia including eastern parts of Indian sub continent, mainly affecting wheat crop (Dubin and van Ginkel, 1991). It is also known as common root rot or dryland rot and may be caused by one or more fungi depending upon the location. Along with *Fusarium graminearum* (Fg), *F. culmorum* and Cochliobolus sativus (*Bipolaris sorokiniana*) are also widely associated in the disease complex. Herein, the pathogen is specialized to parasitise roots and incite necrosis which escalates into rot syndrome. Losses due to root rots incited by a pathogenic complex of fungi in barley are estimated 6 - 20 % (Mathre, 1982).

Seed-treatment with fungicides reduces the spread of seed-borne inoculum and increases seedling vigour (Martin and Johnston, 1982; Teich and Hamilton, 1985). But, cost of seed treatment may act as an impeding factor of farmers’ choice to adopt this method of control. Therefore, integrated disease management approaches, using chemicals as well as eco-friendly host resistance need to be focussed for sustainable barley production in soils infested with root rot pathogens. The present work was undertaken to improve ability to select barley cultivars for root rot resistance in breeding programmes. Herein, attempt to recognise cellular host responses associated with resistance of roots of barley (may be applicable to other cereals such as wheat etc.) to Fg in a bid to find substitute of resistance evaluation based upon screening of genotypes under artificial epiphytotics which is cumbersome, time consuming and sometimes may be misleading owing to disease escapes.

**MATERIALS AND METHODS**

**Host and pathogens**

Seeds of barley genotypes Chevron and Uschi were rinsed in 90% ethyl alcohol for one minute...
followed by disinfection in 6% sodium hypochlorite. Subsequently, seeds were aseptically placed for germination in 9 cm Petri plates on moistened sterile filter paper at 24°C in dark. For root inoculation, 4 - day - old seedlings were used.

Strain of *Fg* known to possess *Tri 5* gene meant for toxin production was used in the study. Pathogen was cultivated for 2 weeks in SNP medium (recipe: 1g KH2PO4, 1g KNO3, 0.5g MgSO4.7H2O, 0.5g KCl, 0.2g glucose, 0.2g saccharose, 14g agar agar in 1 l water) at 25°C in a UV light chamber. The U. V. light stimulated the production of macroconidia used for inoculation of roots. Macroconidia concentration in inoculum solution was adjusted to 50,000 units / ml with the help of a haemocytometer.

**Root inoculation with conidial suspension**

Petridishes containing six 4 - day - old seedlings and 30 ml of spore suspension were gently shaken for 2 hours on the platform of a sea - saw kind shaker. Subsequently, seedlings were placed flat on the sheet of a wet filter paper and covered with another sheet of wet filter paper. The double layer of filter paper enclosing inoculated seedlings was worked to attain the shape of a loosely folded tube. These tubes were placed erect in 30ml Falcon tubes in a manner that epicotyl ends of seedlings could grow upward while incubated in a growth chamber (22°C and 16 - h photoperiod). The filter paper tubes were maintained in a bibulous state by regular filling of water in the Falcon tubes for next seven days. The inoculated roots were repeatedly sampled out from the unfolded tube at 24, 48, 72 and 96 hours after inoculation (hai) for examination of penetrated cells, CWAs and H2O2 accumulation.

**Appraisal of root rot intensity**

Severity of root rot (Fig. 1) was visually estimated after 7 days of inoculation as percent necrosis in total root mass of individual seedlings. Percent necrotic values were assigned using a scale of 0 – 100 following an interval of 10.

This root infection assay was repeated three times by inoculating six seedlings each time and thus using total 18 seedlings. The individual ratings on 18 seedlings (percent necrosis values) were subjected to the analysis of standard deviation for mean.

**Microscopic analysis**

Epifluorescence and bright field microscopy of specimens treated with diaminobenzidine stain
(DAB) and fluorescent brightener calcofluor were employed to reveal the cellular interaction of barley genotypes with *Fg*. Ten root pieces were randomly selected from each sample. Three sites located respectively in the beginning, middle and end of each root piece were examined. Each site essentially had presence of blue stained fungal hypha on the surface. Staining of superficial fungal structures (presence of fungal hypha/e in the vicinity of site showing penetrated cell, CWAs and H$_2$O$_2$ accumulation) were done following Hueckelhoven and Kogel (1998).

**DAB staining technique**

Production of H$_2$O$_2$ at the cellular level was examined by applying DAB stain (Thordal-Christensen et al., 1997) to the roots after 24, 48, 72 and 96 hours after inoculation. The root portion of inoculated plants were fully plunged in 1 ml DAB solution (1 mg / ml distilled water at pH 3.8 – 4 in 2 ml Eppendorf tube) and incubated in light at 22°C for 8 hours.

**Test for specificity of DAB – H$_2$O$_2$ staining**

The specificity of staining was verified by adding ascorbate, an H$_2$O$_2$ scavenger. Separate sets of roots were also plunged in 1, 10 and 50 mM of ascorbate mixed with DAB (1mg/1ml) in the ratio of 1:1 (vol / vol) (Fig. 2 D).

Thereafter, roots treated with DAB and DAB + Ascorbate were cleared, fixed and stored for microscopic observations following procedure of Hueckelhoven and Kogel (1998).

**Calcofluor staining technique**

The roots stained with DAB were cut into 1cm pieces, washed 2 X 15 min with 50 % ethanol, 2 X 15 min with 50 mM NaOH, 3 X 10 min with distilled water and incubated 30 min in 0.1 M Tris HCl (pH 8.5). Subsequently, root pieces were stained for 30 min with 0.3% (w / v) calcofluor and finally washed 4 X 10 min with distilled water. The root pieces were stored in 50% glycerol before their examination under microscope.

**Penetration frequency determined by degree of colonization (fluorescence microscopy)**

The rhizodermal cells that contained intracellular calcofluor stained *Fg* mycelium, irrespective of restricted within a cell, or spreading among cells were regarded as penetrated (Fig. 2A). The intracellular hyphae were examined exploiting the autofluorescent property of calcofluor by UV - fluorescence microscopy.

**Quantitation of CWAs and H$_2$O$_2$ synthesis (bright field microscopy)**

Spherical brown bodies on rhizodermal cell walls present in the vicinity of blue stained hyphae were designated as CWAs which are also known as papillae and are considered as defense structures (Fig. 2B). The cortical cells in a site showing detectable brown stain were regarded as synthesizing H$_2$O$_2$ (Fig. 2C).

**Statistical analysis**

Penetrated rhizodermal cells, CWAs on rhizodermal cells and DAB stained cortical cells were enumerated separately in different sites. Data recorded for each trait in 30 sites were subjected to the analysis of standard deviation about mean.

**RESULTS**

Intensity of root rot recorded significantly higher in Uschi than Chevron after inoculation with *Fg*

Necrotic rot occurred to the tune of 10% and 90% respectively in the roots of Chevron and Uschi after 7 days of inoculation (Fig. 1 and Fig. 3A).

Penetration frequency is directly related to root susceptibility

The roots of Uschi showed the highest number of penetrated rhizodermal cells after 24 hr of inoculation. Also, a gradual increase was recorded in the number of penetrated cells at 48, 72 and 96 hours. Significantly lesser penetrated cells were counted in Chevron at all the time points (Fig. 3B).

Higher levels of CWAs and H$_2$O$_2$ accumulation recorded in Chevron than Uschi

Root tissues of barley genotypes under test reacted to invading hyphae by producing local cell wall swellings designated as CWAs (papillae) in rhizodermal cells seen beneath blue stained superficial fungal hypha/e (Fig. 2B). CWAs appeared conspicuous in roots stained with DAB for the
Fig. 2. Pictorial depictions of histo-chemical host-pathogen interactions in barley roots inoculated with *F.graminearum*

A: Micrographs of penetrated cells; view of calcofluor stained hyphae as visible in uv light at 500X.
B: Cell wall appositions emerge at the site of penetration attempt within 24 hours of inoculation and stain brown with DAB (seen at 300X); cell wall appositions restrict further progression of hyphae which are forced to limit within one cell only as seen at 72 hai.
C: View of root site at 300 X showing cortical cells with and without accumulation of H$_2$O$_2$
D: Deterrence of H$_2$O$_2$ by ascorbate in dose-dependent manner confirms browning of cortical cells and CWAs by H$_2$O$_2$ only.

The DAB polymerization products exhibiting brown colour and indicative of H$_2$O$_2$ formation were found in cortical cells of roots after 24 hours of inoculation in both genotypes under study (Fig. 3D). Frequency of cells showing browning gradually increased in both genotypes through 48, 72 and 96 hours of inoculation. However increase of brown cortical cells during 48, 72 and 96 hours was more pronounced in Chevron than Uschi.

**Ascorbate inhibits H$_2$O$_2$ accumulation – confirmatory test for H$_2$O$_2$ induced browning**

The cortical cells with DAB staining appear dense brown which otherwise appear normal brown under the microscope. Hence quantitation of H$_2$O$_2$ accumulation pertained to dense brown cells only (Fig. 2C). Effect of DAB staining could be noticed with naked eye on the roots treated with DAB and compared with those treated with DAB + ascorbate solutions (Fig. 2D). A gradual decrease in the density of browning of cortical cells occurred when increasing concentration of ascorbate were included into DAB solution. Also, the number of dense brown cortical cells decreased proportionally with the amount of ascorbate infiltrated into roots at all time points after inoculation (data not shown).
**DISCUSSION**

Plants have coevolved with pathogens to develop complex, constitutive and inducible mechanisms to defend against infection (Agrios, 1997). When a plant recognises an invading pathogen, active defense mechanisms are induced which can include a hypersensitive response, accumulation of antimicrobial phytoalexins, synthesis of hydrolases and pathogenesis-related proteins, reinforcement of cell walls through callose deposition and lignification, and activation of defense-related genes (Hammond-Kosack and Jones, 1996). Defense mechanisms in a susceptible plant are induced more slowly than those of a resistant plant, and the time required to induce a variety of defense responses appears to be a key factor resulting in a resistant phenotype (Yang et al., 1997). Keeping this concept of host resistance in mind, we attempted to recognise certain cellular host responses associated with resistance of barley roots to *Fg*. This study aimed to elucidate characteristics of the mechanism determining resistance and susceptibility in barley to an ubiquitous cereal pathogen *Fg*. The information generated in this study may find relevance in the investigative efforts targeted to elucidate mechanism of host resistance in several other crops of agriculture and horticulture importance, since *Fusarium* spp. are root pathogens of a broad range of monocot and dicot plants. The cellular methods standardised in the present study may also be used as procedural substitute of resistance evaluation techniques based upon screening of genotypes under artificial epiphytotics which are cumbersome, time consuming and sometimes may be misleading owing to disease escapes.

Though, *Fg* is known more as a pathogen of head scab which is the major disease of wheat and barley spikes and grains in several countries of the world (Schroeder and Christensen, 1963). But, economic importance of *Fg* increases much more as it also infects roots and causes serious concern

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**Fig. 3.** Results of root infection assay performed by inoculating 4–day–old seedlings of barley genotypes with *F. graminearum*

1% necrosis implied visual assessment of necrotic area on roots of individual seedling by assigning values between 10 – 100 following an interval of 10

2A site comprised a view of root cells seen penetrated by calcofluor stained hyphae (visible in U.V. light) while focussed at 400 X in the vicinity of blue stained hyphae (visible in ordinary light)

3A site comprised a view of rhizoderm cells focussed together at 400 X in the vicinity of fungal hyphae

4A site comprised a view of intensively brown cortical cells among the normal brown cortical cells of root while focussed at 400 X in the vicinity of blue stained hyphae (visible in ordinary light)
to wheat and barley cultivation in the mediterranean area of the middle east, the pacific northwest of the United States (Mathre, 1982) and warmer areas of south Asia (Dubin and van Ginkel, 1991). It is equally, rather more important to protect roots since achieving the full production capability of a crop such as wheat and barley, with maximum fertilizer - use efficiency and ability of the crop to compete with weeds, depends critically on healthy, fully functional roots to support and sustain the plants through all stages of their growth and development.

Conidia or chlamydospore as described commonly for all root pathogens including Fg (Mathre, 1982), germinate in presence of susceptible hosts and initiate primary infection on the coleoptile or on primary roots. Infection then proceeds from the epidermis (rhizodermis in roots) to the cortex and endodermis, resulting in breakdown of these tissues.

Comparison of interactions of two barley genotypes Chevron and Uschi revealed contrasting response to Fg infection in their roots (Fig. 1). The most prominent subcellular structures observed after inoculation of roots were CWAs formed on the surface of rhizodermal cells in the vicinity of invading fungal hyphae (Fig. 2B). These appositions stopped growth of hyphae progressing towards healthy root tissue. The blockade of hyphal progression took place during earlier hours of inoculation (24 hai) and continued even after 96 hours. Sites showing hindered growth of hyphae preventing them from further progression were profusely encountered in Chevron while only meagerly in Uschi. This means CWAs noted to appear in rhizoderm cells after inoculation of roots may have a role in penetration resistance to Fg. Significantly lesser frequency of CWAs in roots of Uschi (Fig. 3C) which showed severe root rot (Fig. 1) and significantly higher number of penetrated cells (Fig. 3B) support the notion of penetration resistance imparted by CWAs.

Induction of H$_2$O$_2$ synthesis in plants, the so called oxidative burst, has not been studied for its role in resistance mechanisms working against Fg infection in roots of barley or any other cereals. The oxidative burst is one of the most powerful defence measures of plants against microorganisms (Lamb and Dixon, 1997). Presently, we studied whether an oxidative burst is also induced during the interaction of Fg with roots of barley. Our earlier observations (Kumar et al., 2001) regarding oxidative burst supporting resistance in Mlo barley against perthotrophic fungus Bipolaris sorokiniana which is also a root rot incitant of wheat and barley (Hill et al., 1983 and Mathre, 1983) prompted us to check the validity of this phenomenon in pathosystem involving barley and Fg.

H$_2$O$_2$, the product of oxidative burst is thought to play a dual role in plant resistance to pathogens. On the one hand, they are involved in induction of several defence reactions, including pathogenesis - related gene expression, phytoalexin synthesis, and the hypersensitive reaction (Huekelhoven and Kogel, 1998; Kumar et al., 2001). On the other hand, their accumulation may be involved in successful pathogenesis (Govrin and Levine, 2000; von Gönner and Schlösser, 1993; von Tiedemann, 1997).

For in vivo detection of H$_2$O$_2$ at cellular level, we used diaminobenzidine (DAB) which yields brown staining at the sites where H$_2$O$_2$ is present (Thordal - Christensen et al., 1997). Using brightfield microscopy we demonstrated specific levels of H$_2$O$_2$ accumulation in susceptible and resistant host reactions. An oxidative burst was locally induced in cortical cells of roots at sites showing presence of ready - to - invade fungal hyphae (blue - stained) on the corresponding root surface (Fig. 2C). Presently, we found H$_2$O$_2$ (tissue browning) in CWAs beneath invading hyphae (Fig. 2 B) and in root cortical cells (Fig. 2C) to be spatially linked to restricted development of Fg in root tissues. The scavenger ascorbate inhibited the colour reaction significantly, demonstrating that tissue browning was due to a chemical reaction involving polymerization of DAB in the presence of H$_2$O$_2$. A gradual decrease of browning in roots (Fig. 2D) inoculated with Fg occurred on elevating the concentration of the ascorbate in the DAB solution.

Cortical cells with H$_2$O$_2$ were enumerated in higher frequencies in Chevron as compared to Uschi. As described earlier, Chevron also supported higher frequencies of CWAs and lower frequencies of penetrated cells in contrast to Uschi. Hence, we suggest that H$_2$O$_2$ is involved in augmenting the inaccessibility of cortical cells. The inaccessibility provided by H$_2$O$_2$ may be attributed to hypersensitive
cell death as has been observed by earlier workers in barley (Hueckelhoven et al., 1999) and lettuce (Bestwick et al., 1998). The hypersensitive cell death has been shown to be a powerful defense mechanism for a number of race/cultivar – specific resistance and many cases of nonhost resistance (Jahnen and Hahlbrock, 1988; Somssich and Hahlbrock, 1998; Kamoun et al., 1999). This type of resistance may act to confine the pathogen, locally activate defense genes, and induce systemic acquired resistance (Pontier et al., 1998).

In the present study, rapid and localized cell death in response to pathogen challenge might have directly prevented the spread of the pathogen into healthy root cortical tissues leading to prevention of root rot in Chevron. It can be further studied whether this event goes in concurrence with the activation of peroxidase and phenylalanine ammonia – lyase as already demonstrated in regulation of defense responses to Fusarium infection in roots of Asparagus densiflorus (He et al., 2001).

ACKNOWLEDGEMENTS

Alexander von Humboldt Stiftung, Bonn, Germany for granting post doctorate fellowship of 6 months’ duration (April – Sept., 2004) to Dr. J. Kumar.

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Received for publication October 13, 2005