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Efficacy of Derelict Water Bodies to be a Fish Culture Pond: A Potential Survey Based on Influence of Physicochemical Parameters on the Bacterial Population and Enzyme Activity

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Abstract

Industrial effluents, agricultural run offs and sewage from households are regularly discharged into the derelict water bodies as well as fish culture ponds, which generates a potential risk for both human and fish. In the present study, one month survey (August, 2019) was conducted in eight different derelict ponds randomly selected from Kalyani subdivision of Nadia district, West Bengal, India to see its potential efficacy to be a fish culture pond by analyzing its physicochemical and microbiological parameters along with enzymatic assay. Different physicochemical parameters like dissolved oxygen, pH, temperature, conductivity, phosphate, nitrate, nitrite, ammonium-N, chemical oxygen demand, hardness, organic carbon and total alkalinity of the water were statistically analyzed with the abundance of heterotrophic and phosphate solubilizing bacteria in the studied ponds. High and low bacterial enzyme activity on the other hand clearly reflected the optimum and unfavouranle nutrient enrichment conditions in water. It was found that the sample data provide strong enough evidence to conclude that the bacteria count have a pressure over water quality parameter studied in different ponds particularly on phosphates and hardness of the water as the P value is less than significance level of 0.05 causing rejection of null hypothesis. The data provides additional information regarding alteration of bacterial enzyme activity in the studied ponds. Thus, the ponds warrant for adoption of proper measures to reduce the pollution level at the point source to be a fish culture pond.

Keywords: Water pollution; Physicochemical parameters; Bacterial load; Bacterial enzyme.

Introduction

In view of the harmful effects caused by the effluents of various industries as well as domestic sewage to the aquatic environment, efforts are now being made to assess the utility of water for human as well as aquatic organisms. A major portion of Nadia district, West Bengal, India located in the basin of river Ganges is marked with a number of derelict wetlands in the form of ponds and ox bow lakes are facing high degree of inorganic and organic pollution from pesticides, jute retting practice, chemical fertilizers from the adjoining agricultural fields and cause a great alteration of the ecological homeostasis of the aquatic eco systems. Inorganic contaminants, in particular heavy metals are known to a prominent environmental concern because they are not biodegradable and can accumulate in living organisms (Fu and Wang, 2011). As water is vital for our existence and its importance in our daily life makes it imperative for the quality test thorough microbiological and physicochemical examination (Chandra et al., 2006; Shittu et al., 2008). Total coliform and faecal coliform counts are known

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to widely used bacteriological procedures for assessment of the quality of drinking and surface water (Mcdaniels et al. 1985). On the other hand, the efficacy of total heterotrophic bacteria and phosphate solubilizing bacteria has been evaluated in earlier studies (Hasan et al., 2012). There are reports that the fluctuating physical and chemical characteristics of water and their interactions bear an effect on the biological features of aquatic ecosystems of rivers (Downing 1971), its catchments (Venter et al., 1997) and watersheds (Guissani et al., 2008), thus prevention of aquatic pollution requires effective monitoring of physicochemical and microbiological parameters (Bonde 1977; Ramteke et al., 1994). Considering the importance of aquatic ecosystem, an assessment of fish health, water quality status and bacteriological studies of the derelict water bodies is of immense importance in order to evaluate its restoration capacity back to its resilient status. Since the affected aquatic nutrient cycle involves changed microbial degradation pathways, it is necessary to employ the microbial signature status of stress levels through assay of nutrient mineralization enzymes such as urease, L-asparaginase, nitrate reductase, nitrite reductase, acid and alkaline phosphatase (Luo et al., 2017). These enzymes catalyze the release of Nitrogen and Phosphorus from organic matter and thus serve as sensitive indicators to pollution induced environmental stress (Brzezinska et al, 2006; Dodds et al., 2010), whose quantitative determination along with bacterial biomass give a clear assessment of alteration of biogeochemical cycling metabolic pathways (Zhou et al., 2005). Meaningfully, bacterial population with their functional attributes has been found to act as an ecological signature of biological integrity to measure the performance and reclamation ability of wastewater ecosystem (Lahiri et. al., 2015)

In this backdrop, the present paper aims to evaluate the influence of physicochemical parameters on the abundance of bacterial load and alteration of bacterial enzyme activity in ponds of Nadia district, West Bengal, India for a better understanding of the ecosystem responses to pollutants and to formulate sustainable prevention measures.

Materials and Methods

Site selection: For the present study, the authors randomly selected eight different derelict ponds based on their eutrophication status, run-off distance from agricultural fields, domestic usage and discharge etc. from both the municipality and block areas from Kalyani Subdivision of Nadia district, West Bengal, India. Fishes are available in all the ponds. The study was conducted during August, 2019 in the following ponds.

Table 1: Details of selected ponds indicating their physical stress status obtained from field survey.

Name of the ponds	Major sources of pollution	Cultivable species of fish
Unused pond at Chakdaha Municipality (P-1)	Agricultural waste, jute retting waste and religious waste.	Rohu, catla, mrigal, grass carp, etc.
Unused pond at Chakdaha Municipality (P-2)	Agricultural field runoffs, religious wastes and dumping of household wastes.	No fish present.
Fish culture pond at Chakdaha Municipality (P-3)	Washing clothes, cleaning household utensils, bathing and runoffs.	Rohu, catla, mrigal, grass carp.
Fish culture pond at Chakdaha Municipality (P-4)	Washing clothes, cleaning household utensils, bathing and runoffs.	Rohu, catla, mrigal, bata, tilapia, etc.
Unused ponds at Dighra under Chakdaha Block (P-5)	Runoffs from surrounding agriculture fields.	Magur, tilapia and other hardy fishes
Fish culture pond at Dighra under Chakdaha Block (P-6)	Household wastewater, bathing (both human and animals), washing clothes and utensils.	Bata, catla, rohu,mrigal etc.
Unused fish pond at Kalyani Municipality (P-7)	Dumping of municipal wastes, idol immersion.	Mrigal, tilapia etc.
Used fish pond at Kalyani Municipality (P-8)	Bathing, washing clothes, runoffs.	Rohu, catla, mrigal

Examination Procedures

Water samples were collected (Rodina, 1972), at regular intervals for physicochemical, bacteriological and enzyme activity study. The surface and bottom samples were pooled separately into single sterilized containers. Two subsamples were taken from each of pooled surface and bottom water samples; one in a properly sterilized 125 ml glass stoppered bottle for enumeration of microbial populations and the other in a 500 ml

plastic sampling bottle for the examination of the physicochemical characteristics of water.

Water quality analysis

Different physicochemical parameters, viz., dissolved oxygen (DO), pH, temperature, conductivity, phosphate, nitrate, nitrite, ammonium nitrogen, chemical oxygen demand (COD), hardness, organic carbon and total alkalinity was measured during study time in all the selected eight ponds following the standard protocols of APHA (APHA, 2017).

Examination of bacterial population

All the routine procedures (Rodina, 1972) were followed for culture of heterotrophic bacteria (HB) and phosphate solubilizing bacteria (PSB) of water samples using conventional spread plate technique (Buck and Cleverdon, 1960).

Qualitative and quantitative assay of microbial enzyme activity

Urease (URE): Bacterial isolate was grown in modified urea broth base (HiMedia) supplemented with 5 ml of 40 % urea solution in 100 ml broth. At every 24h, broth was withdrawn and from its cell free supernatant urease assay was performed till 7 days. The urease activity was measured by phenolhypochlorite assay (Weatherburn, 1967). Controls used for the enzyme reactions were reaction mixture without substrate and reaction mixture without incubation. One unit of urease activity was defined as the amount of enzyme liberating 1 µg NH3 from urea per minute, under the above assay conditions.

L-asparaginase (LAS): In the sample tube, 0.1 ml of enzyme was taken along with 1.0 ml of Tris buffer solution and 0.1ml of asparagine with 0.90ml deionized water. The reaction mixture was incubated at 37°C for 30 minutes. The sample and control tubes were allowed to centrifuge for few minutes to clarify the enzyme.Each tube (sample and control) containing 0.2 ml of supernatant was mixed with 4.30 ml of distilled water and then added 0.5ml of Nessler's reagent. The contents in the tube were mixed by inversion for 1 minute and the absorbance was noted at 436nm.

Enzyme activity (U/ml)=Amount of NH4 liberated* total reaction volume/ (Incubation time × ml of enzyme taken for test).

Statistical analysis

All the data were subjected to statistical analysis (Gomez and Gomez 1984). One way analysis of variance (ANOVA) with the help of MS Excel and computer software SPSS (version 7.5) were used at 1% and 5% levels of probability between all the physicochemical and bacteriological parameters.

Results

A summarize data of physicochemical parameters for all the eight ponds is tabulated in Table 2 and Fig. 1. It was found that among the physicochemical parameters studied, the DO ranges between 4.31 and 7.91 mg/l, pH ranges between 7.2 and 8.6, temperature ranges between 35.7 and 37.10C, conductivity ranges between 463.7 and 1048.8 µSiemens/cm, phosphate ranges between 0.060 and 0.469 mg/l, nitrate ranges between 0.107 and 0.618 mg/l, nitrite ranges between 0.0022 and 0.0101 mg/l, ammonia-N ranges between 0.071 and 0.683 mg/l, chemical oxygen demand ranges between 139.2 and 288.4 mg/l, hardness ranges between 31.72 and 57.34 mg/l, organic carbon ranges between 4.24 and 6.92 mg/l and total alkalinity ranges between 11.25 and 55.73 mg/l in the eight different studied pond (see table 2). On the other hand, the density of heterotrophic bacteria varied from 77 to 158 cfu/100 ml and the density of phosphate solubilizing bacteria ranges between 33 and 62 cfu/100ml in all the studied ponds (Table-3; Figure 2). Maximum urease activity with high concentration recorded on 3rd, 4th, and 6th day in P-1, P-3 and P-4 were shown by HB isolates, whereas comparatively lower activity occurred maximally 3rd day in P-2 for PSB isolates.

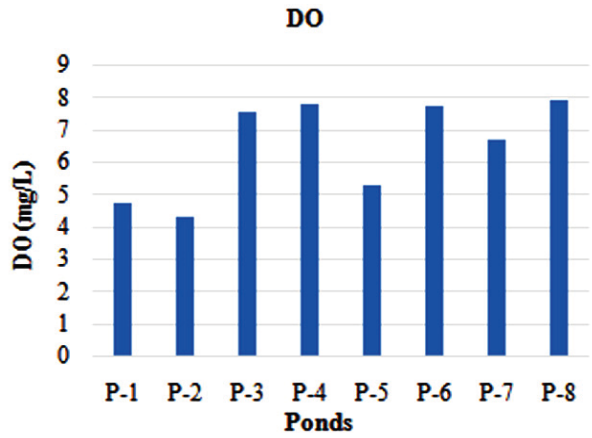
Maximum L-asparaginase enzyme activity with very concentration was shown by HB isolates in P-4 and P-8 whereas comparatively lower enzyme activity was shown by HB in P-2.

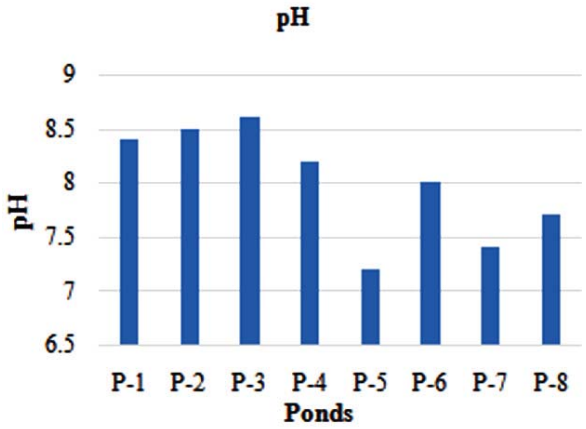
A critical analysis of the data showed that the amount on nitrate and nitrite is very low in all the studied ponds when compared with its standard range. However, the amount of Ammonium-N is considered within the standard value of fish culture ponds except for P-3 and P-4, whereas the hardness in not in the standard range for P-3, P-4, P-6 and P-8. The amount of total alkalinity is found to be lowered in all the ponds except for P-3. The amount of COD in all the ponds clearly indicated

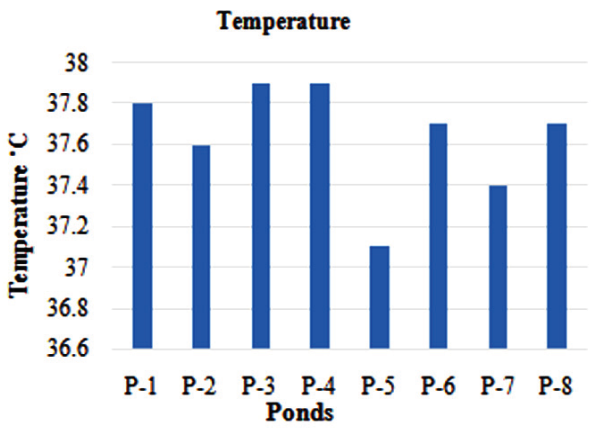
the pollution load in all the studied ponds (Table-2). The trend of the density of both the heterotrophic as well as phosphate solubilizing bacteria is different for different ponds indicated differential bacterial load in the culture ponds (Table-3).

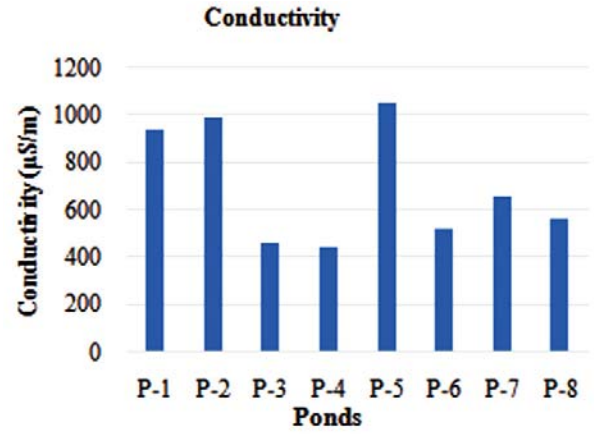
Table 2: Water quality regime of the eight ponds under study (August, 2019).

Studied Ponds	Water Quality Parameters											
	DO (mg/ Lt)	pH	Tempe-rature (˚C)	Conduc-tivity (µS/ cm)	Phos-phate (mg/ Lt)	Nitrate (mg/ Lt)	Nitrite (mg/ Lt)	Ammo-nium -N (mg/ Lt)	COD (mg/ Lt)	Hard-ness (mg/ Lt)	Organic Carbon (mg/Lt)	Total alka-linity (mg/ Lt)
*Standard value for aquac-ulture	>4.0 mg/l	7.5-8.5	Species depend-ent	30–5000 µSiemens/ cm	< 0.5 mg/l	< 100 mg/l	< 1 ppm	0 – 0.5 ppm	2-3 mg/l	40 – 400 ppm	< 10 ppm	50 – 300 ppm
P-1	4.72	8.4	36.8	937.1	0.379	0.141	0.0026	0.343	270.2	57.34	6.05	16.73
P-2	4.31	8.5	36.6	989.9	0.469	0.317	0.0053	0.388	281.5	50.43	4.78	14.18
P-3	7.58	8.6	36.9	463.7	0.076	0.286	0.0119	0.521	146.7	32.66	4.24	55.73
P-4	7.79	8.2	36.9	447.2	0.084	0.618	0.0106	0.683	157.1	31.72	4.95	47.54
P-5	5.29	7.2	37.1	1048.8	0.317	0.372	0.0051	0.418	288.4	40.28	5.33	11.25
P-6	7.74	8.0	36.7	517.2	0.060	0.214	0.0101	0.133	139.2	34.67	5.67	49.36
P-7	6.72	7.4	36.4	661.3	0.327	0.221	0.0022	0.071	211.6	49.33	6.92	16.34
P-8	7.91	7.7	35.7	567.8	0.125	0.107	0.0061	0.116	182.4	37.86	4.28	41.86









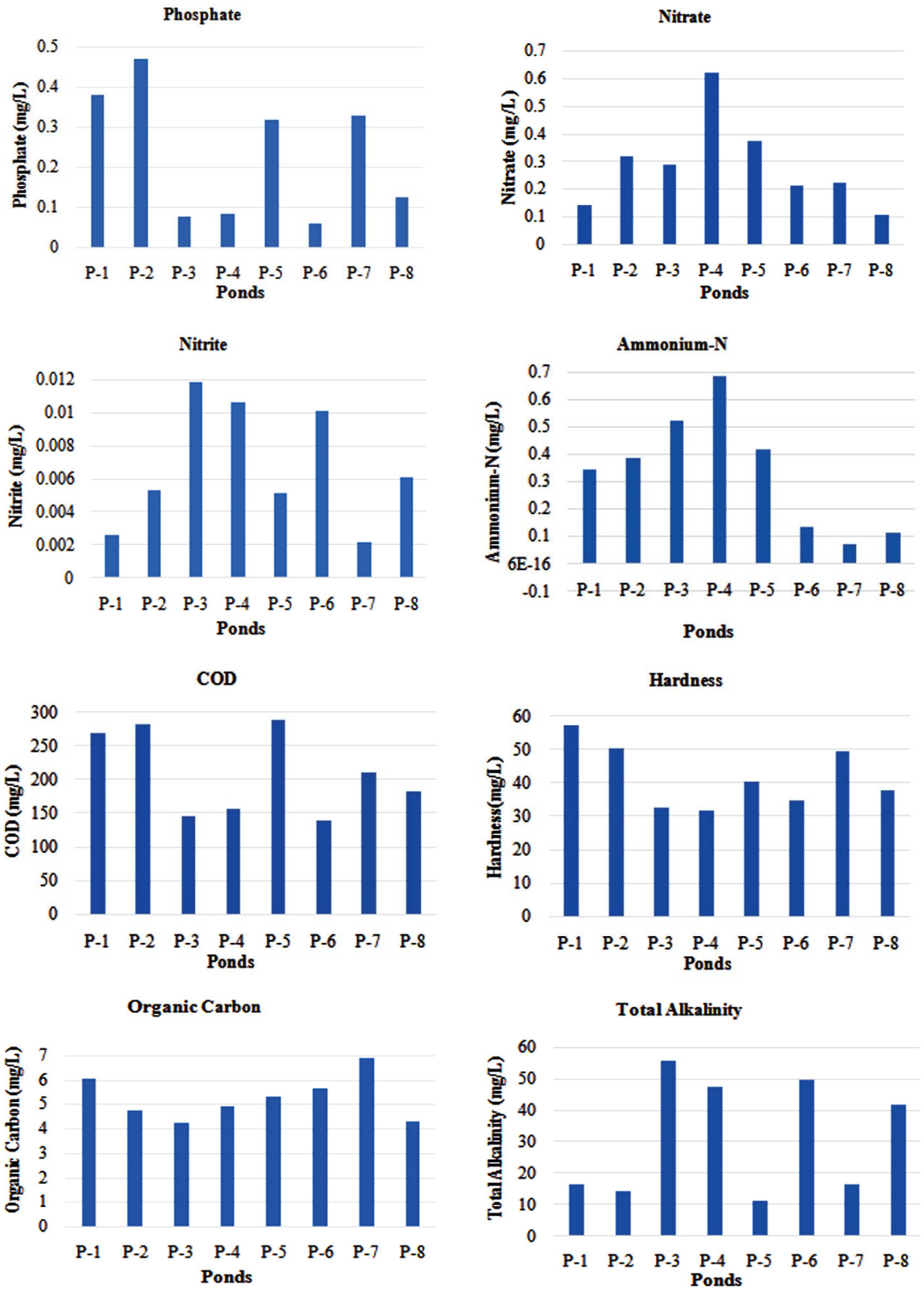


Fig. 1: Graph of all water parameters in the studied ponds.

Table 3: Bacterial counts of heterotrophic and phosphate solubilizing bacteria in eight ponds under study.

Sampling Points	Bacteria count (CFU)	
	Heterotrophic bacteria (×103)	Phosphate solubilizing bacteria (×102)
P-1	117	52
P-2	133	59
P-3	89	41
P-4	77	33
P-5	158	62
P-6	102	55
P-7	126	58
P-8	92	40

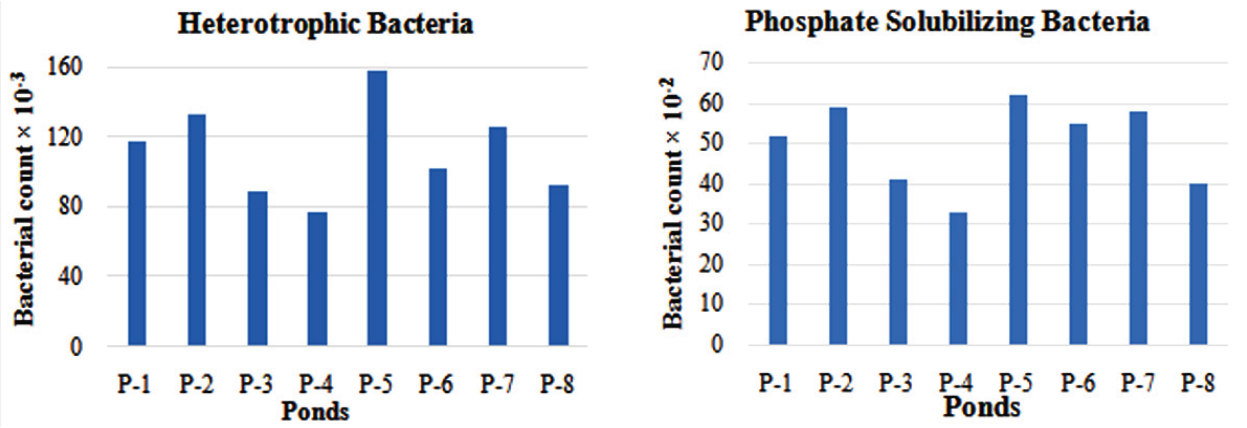


Fig. 2: Graph of bacterial counts of heterotrophic and phosphate solubilizing bacteria in eight ponds under study.

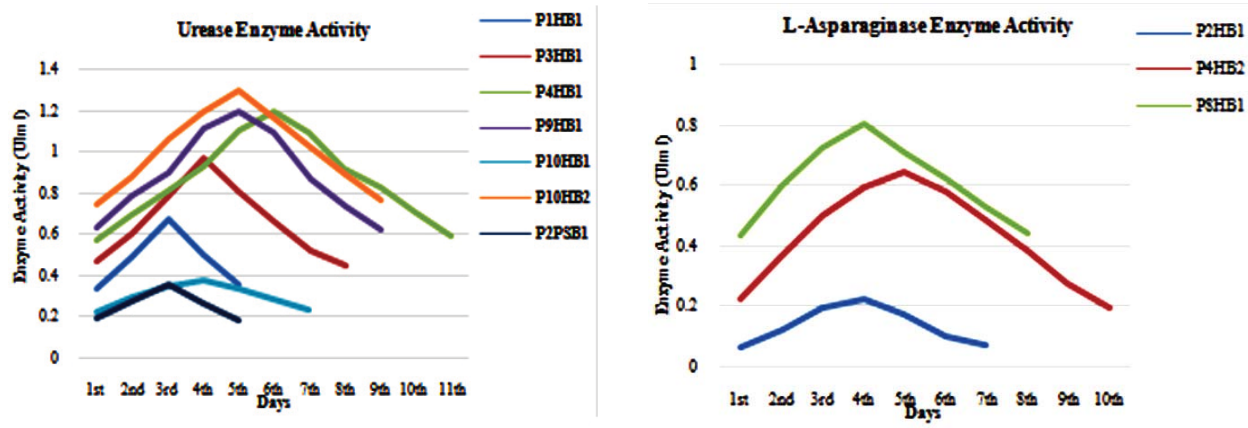


Fig. 3: Graph of Urease and L-asparaginase enzyme activity of the selected bacterial isolates.

Discussion

In the present study, it is evident from the data that different physicochemical and bacteriological parameters of water varied remarkably in eight different ponds under study. It is also found that the studied ponds harbors both the heterotrophic as well as phosphate solubilizing bacteria, the amount was higher than the standard limit of heterotrophic count for drinking water (EPA 2002). In aquaculture, total heterotrophic bacteria (THB) in general and particularly species of Bacillus, Pseudomonas and Lactobacillus provide beneficial effects (Jaganmohan and Prasad, 2010). However, the beneficial effect of using such microbial products in aquaculture is still debatable and controversial as their efficacy is yet unclear. Thus, it can be said that the water of the studied ponds was

polluted in nature and the primary sources of these bacteria in water were mainly animal and human wastes. These sources of bacterial contamination include surface runoff, pasture and other land areas where animal wastes were deposited. Additional sources include seepage or discharge from septic tanks, sewage treatment facilities and natural soil/ plant bacteria (Shittu et al., 2008). As the study was carried out during monsoon time (August, 2019), thus irregular pattern of the occurrence of bacterial count was due to the mixing of some domestic sewerage during the overflowing of runoff water in monsoon time (Chandra et al., 2006). However, optimum level of total alkalinity and low ammonium nitrogen in the ponds indicated higher carbon source, buffering capacity and decay of organic matter respectively (Fast and Lester, 1992). This abundance of the microbes may have a correlation with the physicochemical properties of the water bodies because there are reports that the industrial effluents cause contamination to water during the mixing process (Jain et al., 1996). The slight variations in the physicochemical properties in the studied ponds were noted during monsoon seem to be related with the run off of organic matter into ponds from adjacent river or possibly due to the precipitation factors (Chatterjee et al., 2010; Jain et al., 1996). Nutrient concentration, COD and DO values favored balanced nutrient cycling with biological integrity resulting in high enzyme activity in P-3 and P-4, which are also been used for fish culture. This indicates regulated discharge of waste in these ponds. However, high concentration of phosphate and COD with high population of PSB and low DO in P-2 did not support optimum bacterial metabolism due to its nutrient enrichment condition because of very high dumping of households and other wastes. No fish has been found due to this in P-2 pond.

In ANOVA one way factor analysis between the physicochemical and bacteriological parameters, the experiment indicates that the sample mean is different. The data further support the notion that the population means are not equal and it might be the result of random sampling error. The sample data provide strong enough evidence to conclude that the bacterial count (both HB and PSB) have a pressure over water quality parameter studied in different ponds particularly on phosphates and hardness of the water as the P value is less than significance level of 0.05 causing rejection of null hypothesis.

The present findings seem to be influenced by the domestic and agricultural refusals of the study ponds. Moreover, high COD values in all the ponds indicate the presence of contaminants and bacterial load in the water. Although the nitrite, nitrate, alkalinity and hardness levels are obeying the standard value of aquaculture and the optimum level of DO, pH and temperature amply speaks for suitable fish culture, but the altered bacterial metabolism in the nutrient cycle as indicated by the enzyme activity responses reflected lack of biological integrity of the aquatic ecosystem influenced by the physicochemical water quality parameters. Therefore, the entire study can be applied for as a molecular biomarker of nutrient enrichment status to call for science and technology intervention before making them suitable for any fish culture.

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Conclusion

In search of suitable fish culture ponds for increased production of fish throughout the globe, the study of physicochemical parameters and their influence on the the bacterial counts and bacterial enzymatic assays with their feddback mechanism is of immense importance. Although there are other valid parameters to study, the present results depict that physicochemical environment induced bacterial growth and activity varied remarkably among different derelict bodies with different magnitude of nutrient status, although they are randomly selected from same geographical location. The result depicts that the water of the studied ponds was polluted in nature and the primary sources of the bacteria in water were mainly animal and human wastes and/or due to varied anthropogenic origin of the ponds. The altered bacterial metabolism in the nutrient cycle as indicated by the enzyme activity responses reflected lack of biological integrity of the aquatic ecosystem influenced by the physicochemical water quality parameters in the studied ponds. Therefore, the entire study can be applied as a molecular biomarker of nutrient enrichment status for searching the efficacy of derelict water bodies to be a fish culture pond.

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Induced Temporal and Spatial Variation in Pathogenesis Related Proteins in Cicer Arietinum Inoculated with *Fusarium Oxyporum F.SP. Ciceri*

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Abstract

Pathogenesis-related proteins (PRs) are a class of proteins that accumulate in response to biotic and abiotic stresses to protect plants from damage. The present study examined the defense response of Chickpea (Cicer arietinum L.) wilt resistant and susceptible genotypes inoculated with wilt pathogen Fusarium oxyporum f.sp. ciceri (Foc). Evaluation of pre-induced and pathogen-induced defense at 3 stages i.e. 7 (S1), 15 (S2) and 30 (S3) days showed that the PRs (i.e. β-1,3-glucanase and chitinase) differed not only among the root, stem and leaves but also among susceptible and resistant genotypes and increased after inoculation with Foc. Foc inoculation induced β-1,3-glucanase and chitinase activity in all the test cultivars. Maximum induction of chitinase was observed at S2 in roots of resistant cultivars whereas un-inoculated plants showed much less conspicuous changes. β-1,3-glucanase activity was high in stem tissues. Both control and Foc inoculated plants had higher level of β-1,3-glucanase activity at S2 and S3, but the resistant cultivars recorded much higher proportionate increase.. The activity/expression pattern of these PR proteins could be used as established resistance markers and for manipulating their expression towards development of superior wilt-resistant chickpea genotypes.

Keywords: Chickpea; Cicer arietinum; Fusarium oxyporum f.sp. ciceri; Pathogenesis; PR proteins, chitinase, β-1,3-glucanase.

Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n=2x=16 with genome size of 738 Mb) leguminous crop, cultivated mainly in semiarid environments of the world and ranks second in area and third in production among the pulses worldwide. Around 65% of the total global area and around 68% of total global production of chickpea falls in India. Although much progress has been made in developing chickpea lines with resistance to biotic constraints and tolerance to abiotic stresses, yield loss in this crop is very high due to the high incidence of diseases and insect pests. Chickpea wilt caused by *Fusarium oxysporum Schl*.Emend.Snyd and Hans f.sp. *ciceri* (Padwick) is widespread in several chickpea growing regions of the world, especially the Indian subcontinent, Ethiopia, Mexico, Spain and Tunisia (Joshi *et al.*, 2001). The wilt pathogen is both soil and seed borne and difficult to eradicate as fungal chlamydospores survive in soil up to six years even in the absence of host plant. *Fusarium* wilt epidemics can be devastating to individual crops and cause up to

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100% loss under favourable conditions.

Effector-triggered immunity (ETI) is an important part of plant innate immunity that protects against pathogen infections and is established via the recognition of virulence effectors by the corresponding receptor resistance (R) proteins in a specific gene-to-gene manner (Zhang *et al.*, 2018). These PRs have diverse functions, contributing to cell wall rigidification, signal transduction, and antimicrobial activities, and are mainly expressed in plants as chitinases, glucanases, and thaumatin-like proteins (Farrakh *et al.*, 2018). The PRs are usually small (10–40 kDa) and mostly acidic. Many PRs are distributed in plant cell gaps and vacuoles, and their distribution is related to their isoelectric point and the exposure to stress (Jo et al., 2020). Therefore, they are able to accumulate in intracellular and intercellular spaces. During a pathogen attack, the ETI induction mechanism in plants is activated, leading to the accumulation of pathogenesis related proteins (PRs) (Mazumder *et al.*, 2013). The accumulated PRs help plants prevent reinfections, resulting in the development of systemic acquired resistance (Zhang *et al.*, 2013).

Developing effective strategy for management of wilt diseases demands deep understanding of the molecular basis of pathogenesis and resistance mechanism. Plants activate a large array of defence mechanisms in response to pathogen attack and host pathogen cross talk. A crucial factor determining the success of these mechanisms is the speed of their activation which needs thorough understanding of how host plants recognize pathogen attack and control expression of defence mechanisms. The hypersensitive response of plants is accompanied by a combination of a number of host enzymes involved in immune systems. Defence-related enzymes form an important immune system that fights pathogen attacks as PAL is a key enzyme in phenylpropanoid metabolism and plays an important role in the synthesis of secondary defense-related chemicals called phytoalexins (Tahsili *et al.*, 2014). Elucidation of host defensive responses to pathogen invasion have determined "pathogenesis related" proteins (PRs) viz. chitinase and β-1,3-glucanase as vital defense related enzymes of plants against phytopathogens (Dehgahi et al., 2015). The use of β-1, 3-glucanase is suggested as a biomarker for genotypes that are resistant to downy mildew in pearl millet (Pennisetum typhoides) due to the activity of enzyme differentiation between resistant and genotypes (Ramachandra *et al.*, 2000). Giri *et al.* (1998) recorded differential induction of chitinase in susceptible and resistant genotypes of chickpea and decrease in β-1,3-glucanase in the resistant cultivars in response to the pathogen infection. Significant decrease in β-1,3-glucanase in cultivars resists the pathogen invasion due to a protective response. Expression of a number of enzymes involved in a participatory immune system may be constitutive while others are induced upon the attack of pathogen such as enzymes involved in phytoalexin biosynthesis or antioxidant supplementation and its enzymatic-related system.

Since details on pre-induced and induced biochemical mechanisms of resistance are pre-requisite to understand interaction between pathogens and host and provide basis for better disease management, the present research work attempted to correlate and analyse the biochemical basis of wilt disease resistance in resistant and susceptible cultivars of chickpea by measuring spatial and temporal levels of chitinase and β-1,3-glucanase.

Material and Methods

Plant material, pathogen inoculation and sample collection for biochemical analyses

Two each of wilt resistant (WR 315, ICC 4958) and wilt susceptible (JG 62, BG 256) cultivars of chickpea (*Cicer arietinum* L.) were used for the present study. The *Fusarium oxysporum* f. sp. ciceri (Foc) pathogens were isolated from fourth node stem sections taken from wilted chickpea plants according to the procedure described by Tullu *et al.* (1998) and were colonized on filter paper, dried in the transfer hood, and aseptically cut into small pieces. The colonized filter paper pieces were placed in potato dextrose broth and incubated to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheese cloth to remove mycelia. The spore suspension was pelleted by centrifugation. After discarding the supernatant, the conidia were washed with sterile water to adjust the spore suspension to 1 x 10^6 spores ml-1 with a haemocytometer. Single spore culture of fungus was obtained by serial dilution method. Isolated fungus was identified as *F. oxysporum* f. sp. ciceri and its pathogenicity test was conducted in pot experiments on chickpea cultivar, JG 62. Plastic pots of 30 cm diameter, surface sterilized with 0.1% w/V mercuric chloride ($HgCl_2$), were filled with 2 kg sterilized soil (three subsequent sterilizations at 1.1 kg/cm^2 for 1 h for 3 days). Seven days before sowing, pots were inoculated with the 14 day old culture of the pathogen multiplied on sand maize meal water medium (90 g and, 10 g maize meal

and 20 ml distilled, sterilized water) @ 50 g kg–1 soil. Ten seeds (surface-sterilized using 2% sodium hypochlorite for 3 min, and rinsed in sterile water) of each cultivar were sown in each pot for disease scoring.

The root, shoot and leaf tissues were collected separately at 7 (S1), 15 (S2) and 30 (S3) days after sowing (DAS) and were frozen immediately in liquid nitrogen to store at -20°C. Biochemical basis of disease resistance was studied (temporal and spatial accumulation and activity) for PR-proteins (chitinase, and β-1,3-glucanase) in *Fusarium* wilt resistant and susceptible chickpea genotypes.

Isolation and activity assay of chitinase (EC 3.2.1.14)

Defatted and depigmented tissue powder was stirred with extraction buffer (1:6 w/V in 0.1 M phosphate buffer, pH 6.9 containing 0.05 M NaCl) at 4°C for 12 hrs. The mixture was centrifuged at 10,000 X g for 20 min and the proteins form the supernatants were precipitated by adding ammonium sulphate [$(NH_4)_2SO_4$] to 90% saturation (60 g ammonium sulfate per 100 ml extract). The precipitated protein were collected by centrifugation, resuspended and dialyzed against the extraction buffer (Giri *et al.*, 1998).

Chitinase activity was determined as described by Chen *et al.* (1982) and Tsukomoto *et al.* (1984). The reaction mixture contained 1.0 ml of colloidal chitin solution (7 mg), 1.0 ml of sodium acetate buffer (50 mM, pH 5.2) and 1.0 ml of suitably diluted enzyme. After incubation at 50°C for 1 hr, the released reducing sugar was measured as N-acetyl glucosamine (NAG) equivalents by the method of Reissig *et al.* (1955). One unit (U) of chitinase activity is defined as the amount of enzyme that produces 1 mg of NAG per hour, under the given assay conditions and expressed as U per gram fresh weight (U gfw-1).

Isolation and activity assay of glucanase (EC 3.2.1.39)

β-1, 3-glucanase activity was estimated using the procedure of Koga *et al.* (1988). The assay mixture contained 1.0 ml of suitably diluted enzyme and 1.0 ml of 1% laminarin solution in sodium acetate buffer (50 mM, pH 5.2).The mixture was incubated at 40°C for 30 min and the released reducing sugar was measured as glucose equivalents (Somogyi, 1952). One unit (U) of β-1, 3-glucanase activity is defined as the amount of enzyme that produces 1 mM of glucose ($C_6H_{12}O_6$) per hour, under the given assay conditions.

Results and Discussion

Spatial and temporal activity of chitinase

Induction of chitinase in chickpea was significantly high in leaves. Chitinase activity showed a marked increase in different tissues upon inoculation with *Fusarium*. In general the maximum chitinase activity was observed at S2 stage in resistant cultivars. The chitinase activity increased progressively from S1 to S2 stage and thereafter showed decrease with progression of the disease and was very low at S3 stage (Table 1).

Table 1: Chitinase activity (U gfw-1) in different tissues of chickpea genotypes differing in susceptibility to *Fusarium* wilt.

Genotype	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
WR 315	Control	3.21 ± 0.01	3.23 ± 0.01	3.19 ± 0.02	3.66 ± 0.04	4.06 ± 0.10	3.93± 0.01	2.63 ± 0.02	3.22 ± 0.02	3.16 ± 0.02
	Foc	3.49 ± 0.03	9.96 ± 0.10	8.77 ± 0.01	4.00 ± 0.02	9.95 ± 0.04	9.71 ± 0.01	3.29 ± 0.00	10.15 ± 0.05	9.96 ± 0.04
ICC 4958	Control	3.16 ± 0.01	4.45 ± 0.01	4.41 ± 0.01	2.67 ± 0.01	3.09 ± 0.01	3.07 ± 0.00	3.95 ± 0.02	3.35 ± 0.02	3.36 ± 0.01
	Foc	3.42 ± 0.03	10.40 ± 0.05	10.41 ± 0.05	3.17 ± 0.08	10.62 ± 0.01	10.23 ± 0.00	4.13 ± 0.01	11.72 ± 0.08	11.81 ± 0.00
BG 256	Control	1.51 ± 0.08	4.68 ± 0.00	4.51 ± 0.01	3.19 ± 0.00	5.84 ± 0.05	5.46 ± 0.00	3.29 ± 0.05	4.68 ± 0.00	4.62 ± 0.00
	Foc	4.70 ± 0.04	8.95 ± 0.08	5.28 ± 0.03	4.90 ± 0.04	9.10 ± 0.05	6.54 ± 0.04	5.65 ± 0.05	8.84 ± 0.05	4.74 ± 0.01
JG 62	Control	4.34 ± 0.03	4.57 ± 0.04	4.50 ± 0.00	4.17 ± 0.00	4.39 ± 0.03	4.42 ± 0.03	3.99 ± 0.02	4.24 ± 0.05	4.20 ± 0.00
	Foc	5.22 ± 0.01	9.12 ± 0.15	6.86 ± 0.01	5.63 ± 0.15	8.80 ± 0.00	7.48 ± 0.00	5.94 ± 0.02	9.50 ± 0.05	7.94 ± 0.01

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3, Severe disease stage (30 DAS). All values are mean of three replications ± SD.

On the contrary, these changes were much less conspicuous in case of un inoculated plants, where the increase or decrease was only marginal.

At S1 stage, maximum constitutive level of chitinase activity was observed in leaf, stem and root tissue of susceptible cv. JG 62. The enzyme activity in these tissues was 4.34, 4.17 and 3.99 U gfw-1, respectively (Table 1). Maximum induction of chitinase activity was observed at S2 stage in the root tissue. In resistant cultivar WR 315 this increase was 3.08 fold and in cv. ICC 4958 increase was 2.3 fold. This increase in activity was lower in susceptible cultivars (1.9 fold only). The enzyme activity significantly increased in comparison to control in the stem tissue of resistant genotypes. More than three-fold increase in enzyme activity was observed in resistant plants compared to respective controls but in susceptible cultivars this increase was only 1.18 and 2.24 fold. At S3, in both control and inoculated plants the increase in activity was less pronounced but resistant cultivars showed higher activity of chitinase than susceptible cultivars.

Spatial and temporal activity of β-1,3-glucanase

Glucanase activity was higher in stem and leaf tissues as compared to root tissue. Inoculation of the plants with *Fusarium* recorded a significan increase in glucanase activity in various tissues. In general the maximum enzymatic activity was observed in resistant cultivars at S2 stage and the same was more or less maintained until S3 stage. In case of chickpea, at S1 stage maximum (0.67 mM glucose $h^{-1}gfw^{-1}$) constitutive level of glucanase activity was observed in BG 256 roots. Minimum activity (0.30 mM glucose $h^{-1}gfw^{-1}$) was found in WR 315 (Table 2). At disease initiation stage (S2) maximum induction of enzymatic activity was in resistant chickpea genotype WR 315, where this increase was three times greater in root and stem compared to control plants of same stage. While, under similar condition, in wilt susceptible chickpea genotypes, the increase in enzymatic activity was only 1 to 1.5 fold.

Table 2: Glucanase activity (mMh-1 gfw-1) in different tissues of chickpea genotypes differing in susceptibility to Fusarium wilt.

Genotype	Treatments	Root			Stem			Leaf		
		S1	S2	S3	S1	S2	S3	S1	S2	S3
WR 315	Control	0.30 ± 0.00	0.43 ± 0.00	0.44 ± 0.01	0.52 ± 0.03	0.69 ± 0.00	0.40 ± 0.01	0.34 ± 0.01	0.62 ± 0.00	0.77 ± 0.01
	Foc	0.50 ± 0.01	1.34 ± 0.02	1.29 ± 0.01	1.25 ± 0.02	2.00 ± 0.07	2.29 ± 0.05	0.62 ± 0.01	1.47 ± 0.07	1.73 ± 0.03
ICC 4958	Control	0.46 ± 0.02	0.74 ± 0.03	0.59 ± 0.00	0.90 ± 0.04	0.97 ± 0.01	0.55 ± 0.00	0.95 ± 0.00	0.84 ± 0.01	0.82 ± 0.05
	Foc	1.43 ± 0.00	1.35 ± 0.01	0.96 ± 0.00	0.32 ± 0.02	1.85 ± 0.02	0.80 ± 0.14	0.59 ± 0.01	1.90 ± 0.00	1.88 ± 0.00
BG 256	Control	0.67 ± 0.00	0.74 ± 0.01	1.06 ± 0.01	0.76 ± 0.09	0.96 ± 0.03	0.9 6 ± 0.02	0.47 ± 0.02	0.80 ± 0.00	0.64 ± 0.02
	Foc	1.02 ± 0.00	0.92 ± 0.03	0.39 ± 0.02	0.81 ± 0.03	1.00 ± 0.01	0.96 ± 0.01	0.72 ± 0.17	0.96 ± 0.03	0.57 ± 0.02
JG 62	Control	0.46 ± 0.02	0.96 ± 0.04	1.03 ± 0.01	0.30 ± 0.01	0.79 ± 0.09	0.96 ± 0.01	2.18 ± 2.40	0.57 ± 0.05	1.42 ± 0.14
	Foc	0.50 ± 0.08	1.00 ± 0.00	0.71 ± 0.02	1.017 ± 0.01	0.83 ± 0.00	0.81 ± 0.00	1.14 ± 0.01	0.92 ± 0.00	0.92 ± 0.00

S1: Pre infection stage (7 DAS), S2: Disease initiation stage (15 DAS), S3, Severe disease stage (30 DAS).
All values are mean of three replications ± SD

Induction of plant protection against pathogen invasion is controlled by a complex network of different signals. In the present study, pathogen-host interaction improved immune responses in resistant cultivars of chickpea. Spatial and temporal changes in various enzymes investigated in *Fusarium* infected and uninoculated plants showed that induction of plants' own defence system program began only after the infection by respective pathogen, and subsequently led to hypersensitive reaction conferring resistance. Chickpea plants exposed to *Foc* showed enhanced synthesis of pathogenesis related proteins relative to their controls.

Inhibition of growth of several fungal pathogens requires the presence of chitinases and β-1,3-glucanase activities to reduce the polymerization of cell wall polymers (Saikia *et al.*, 2005). Ferraris *et al.* (1987) found that infection of susceptible and resistant tomato cultivars with *F. oxysporum* f. sp. *lycopersici* caused several fold increases in chitinases, β-1,3-glucanases, glucosidases and N acetyl glucosaminidases activities between 5 to 90 days after inoculation. The present study in chickpea proved that chitinase activity was increasing 3.08 and 2.33 fold in root tissue, 2.45 fold and 3.43 fold in stem tissue, 3.15 fold and 3.5 fold in leaf tissues of chickpea resistant cultivars while in susceptible cultivars it increased up to 2 fold upon infection. Similarly, in case of β-1,3-glucanases, the activity increased by 2-3 fold in resistant cultivars and 1-1.5 fold in susceptible varieties of chickpea. Giri *et al.*

(1998) analysed the levels of these two enzymes in resistant and susceptible cultivars of chickpea during *Fusarium* wilt development and found induction of chitinase activity in both resistant and susceptible cultivars; however induction in susceptible cultivar JG 62 was much lower than that of resistant cultivar. Further, the activities of these two enzymes increased several fold from S1 to S2 stage both in control and treated condition and declined thereafter. The induction was very low in the control condition. This fact is also supported by work of Saikia *et al.* (2005) where the radial growth of different fungal species, e.g. *F. oxysporum.* f. sp. ciceri, *F. udum* and *M. Phaseolina* was inhibited by purified proteins, thus exhibiting antifungal activity. Chitinase exhibited more antifungal activity in comparison to β-1, 3-glucanase *in vitro*. Rakshit *et al.* (2000) reported that increase in β-1, 3-glucanase activity was significantly higher and more severe in resistant genotypes as compared to the susceptible ones in pea after powdery mildew infection. Xue *et al.* (1998) observed significantly higher activity of chitinase in the cotyledons of bean plants than hypocotyls and a significant increase of peroxidase, β-1, 3-glucanases and chitinase in all fractions of diseased plant cells as compared to control, and β-1, 3-glucanases activity recorded increase up to 18 fold in induced bean hypocotyls tissues. Similar increases, but not as pronounced have been reported during ISR studies using incompatibility interactions between soybean and *Phytophthora megasperma* f. sp. *glyciensi* (Yi and Hwang, 1996) and between resistant bean cultivars and *C. lindemuthianum* (Daugrois *et al*., 1990). In the present study, chitinase accumulated significantly in different tissues after the onset of disease and the same has been reported in other systems of host pathogen interactions where different types of chitinases are expressed depending on the type of plant tissue and developmental stages (Igratius *et al*., 1994). Seed associated barley chitinases differ from those found in leaves infected with powdery mildew (Igratius *et al*., 1994) while Anuratha *et al.* (1996) isolated and identified infection related chitinase transcripts that were only induced after infection of rice with the sheath blight pathogen *R. solani*.

The defence responses were induced in both pre induced and non induced plants infected by pathogens and the suppression of *Fusarium* wilt possibly involved an inhibitory effect on the pathogen of pre-induced plant defences, as well as induced defence. Chickpea resistant cv. ICC 4958 did not show significant increase in activity of β-1, 3-glucanase in root tissue at S2 stage as compared to susceptible cultivars. The increase in activities in susceptible hosts may have been a result of failure of containment of the pathogen and the colonization of ever increasing amounts in the vascular tissue. Benhamou *et al.* (1990) observed faster accumulation of defence related enzymes in incompatible interactions of tomato and *Fusarium oxysporum* f. sp. *lycopersici* or *Fusarium radicis lycoperscici* than in a compatible interaction. Beckman and Roberts (1995) in their model for host-pathogen interaction, suggested callose deposition and lignifications as one of the plants responses against wilt disease and rate of these processes determining the degree of reduction of pathogen invasion. The observed decrease in β-1, 3-glucanse activity in resistant cultivars in root and stem tissues may be associated with the higher rate of callose insertion. When expression of β-1, 3-glucanase is specifically blocked by the antisense mRNA technique, callose deposits are protected from degradation, leading in resistance to viral infection in tobacco (Beffa *et al*., 1996). In the present study, levels of chitinase and β-1, 3-glucanases are reduced as the infection is lasted at S3 stage. This decrease may be associated with the reduction in the pathogen attack due to a protective response. In summary, this study shows that *Fusarium* isan inducer of these enzymes at both the local and systemic level in tissues of chickpea. Induced resistance is multi-component and it is neede to investigate further other mechanisms involved, either individually or collectively and focus on identification and transfer of disease resistance genes as part of an integrated wilt management strategy.

Conclusion

The present research study on temporal and spatial variation in PR proteins reveals that their expression pattern could be used as biomarkers to establish resistance. The host is protected against pathogen both by passive (physical barriers) and active (phytoalexins) mechanisms using defense enzymes. The isolation of pathogen induced defense enzyme genes bears scope for their cloning, manipulation of expression and development of transgenic crop plants resistant to various pathogens.

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Review Article

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Climate Change and Apple Cultivation in India

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Abstract

Apple crop is highly sensitive to weather stress. Temperature plays an important role on successful production of apple in temperate regions. This crop requires very specific temperature at its different stages. The average summer temperature should be around 21-24oC during active growth period. Apple succeeds best in regions where the trees experience uninterrupted rest in winter and abundant sunshine for good colour development. It can be grown at an altitude of 1500- 2700 m above the sea level. Bud dormancy, break and development are affected by variation in temperature. The amount of cold needed by a plant to resume normal spring growth following the winter period is referred to as its "chilling requirement." Buds remain dormant until they have accumulated sufficient chilling units of cold weather. Apple also requires accumulated heat units for onset of flowering. Mean maximum temperature in February, March and April is found to be most important variable in apple yield prediction in Indian conditions. Besides, above ground temperature, variations in soil thermal regimes also impact on tree physiology. Fruiting is also a temperature driven process. Frost can damage the full bloom stage up to 60-70% crop when temperature is around -2.2OC. Spring frost is not damaging as much as winter frost. In the changing climatic scenarios, apple production in Uttrakhand is unquestionably going to be affected in forthcoming days.

Keywords: Apple; Climate Change; Global Warming; Future Prospect.

Introduction

Apple cultivation and weather

A prospective apple farmer shall make an extensive research regarding the variety that most closely matches the climate conditions of his/her region. Apple is temperate deciduous thermal sensitive long day plant. It is one of the important fruit in the temperate regions. It is mainly cultivated with in the range of elevation of 1350-2700 meter above the mean sea level. Well distributed rainfall of 1000-1250 mm throughout the growing season is most favourable for optimum growth and fruitfulness of apple trees. Ignoring the quality of the harvested product substantially biases the impact of weather extremes on agricultural income and the potential effects of climate change. (Dalhaus, *et. al.*, 2020).

Soil: Apples grow best on a well drained, loam soils having a depth of 45 cm and a pH range of pH 5.5-6.5. The soil should be free from hard substrata and water logged conditions. Soils with heavy clay or compact subsoil are to be avoided.

Apple cultivation is confined to northwest (Jammu and Kashmir & Hinanchal Pradesh), north (Kumaun Hills) and north east (Arunanchal Pradesh) in India. The crop is highly sensitive to weather stress and accounts for limiting factors in its survival and production.

The average summer temperature should be

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around 21-24^{O}C during active growth period. Apple succeeds best in regions where the trees experience uninterrupted rest in winter and abundant sunshine for good colour development.

Nevertheless its growth and development are influenced by many weather parameters; the temperature is identified as one of the most important factors which affect its various phonological developmental phases. The rate of growth and development of a crop is a function of the energy receipt and thermal regime. The consequences of global warming in the form of erratic precipitation, increase in temperature, lesser days serving as the chilling period have started affecting the mountain agricultural production systems and ultimately the food security of the people.

Therefore agrometeorological conditions in growing apple is one of the most important aspects for its proper cultivation. Besides this apple fruit set being, heavily dependent upon insect pollination, is indirectly affected by vagaries of weather.

Climate change effect on Apple cultivation

Global warming put direct or indirect impact on crop, soil, livestock, and overall yield of plant. Apple production is directly affected by temperature, water availability, solar radiation, air pollution, and carbon dioxide (CO_2). Walthall *et al.* (2012) document current and projected shifts in climate patterns and weather and their impacts on United States (U.S.) agricultural production. It was four climate risk is an additional risk joining production, finance and marketing risks already managed by growers. Climate risk will add complexity and increase uncertainty in decision environments throughout many dimensions of U.S. apple production systems. The population of pollinators such as honeybees and butterflies had declined due to high temperature at higher hills which has greater impact on apple production. Due to lack of lower temperature, trees fail to enter into bud development and flowering causing potentially death of tree (Aditya *et al.*, 2012). According to recent reports, temperature has increased by 1.5^{o}C which is above the pre industrial level (IPCC, 2018).

As climate and weather become more variable, apple growers face increased uncertainty in making decisions about their crop. One interpretation of this uncertainty is that growers may not have quite enough information to adequately evaluate their management options in the context of climate risk. Uncertainty can stem from social, economic, relational and/ or biophysical factors that constrain or limit knowledge needed to make timely, good decisions.

Requirement of chilling units

It requires temperature below 7^{0}C for 600-900 hrs. which is called chilling period. This chilling period is required for growth, bud initiation, blossom and fruiting.

The chilling hour's requirement for standard apple variety is 800-1100. The amount of cold needed by a plant to resume normal spring growth following the winter period is commonly referred to as its "chilling requirement." One chilling unit is equal to one hour's exposure to the chilling temperature; these units are summed up for a whole season. It is still true that the most popular varieties need certain hours of cold in order to produce fruits for the next 30-40 years. Advanced models assign different weights to different temperature bands. The daily temperatures of 70°F and higher for 4 or more hours received by the plant during the previous 24 to 36 hours can actually negate chilling.

Apple trees develop their vegetative and fruiting buds in the summer and as winter approaches; the already developed buds go dormant due to both shorter day lengths and cooler temperatures. These buds remain dormant until they have accumulated sufficient chilling units (CU) of cold weather. The buds are ready to grow in response to warm temperatures only when enough chilling units accumulate. As long as there have been enough CUs the flower and leaf buds develop normally. However, in case the buds are unable to receive sufficient chilling temperatures during winter to completely release dormancy, trees will develop one or more following symptoms chilling consequently affect the yield and quality of the fruit:

- delayed foliation
- reduced fruit set and increased buttoning
- reduced fruit quality.

Temperature in relation to apple cultivation

Temperature requirement

Temperature is one of the most important factors for growth of any crop, especially to temperate region crops, though it can not be altered much in field conditions. However, modified orchard management can be used to capitalize to minimize

the unfavorable effects. Certain amount of temperature units are necessary to attain and complete each of the different phenophase which is termed as "thermal unit" or "heat units". The crop remains in dormancy from October to March and bud beak takes place from last week of March to first week of April after completing the chilling requirements. Mean maximum temperature in February, March and April is found to be most important variable in apple yield prediction in Indian conditions. Chilling units, chilling hours, number of hours with temperature more than 18^0C during dormancy temperature during bloom and relative humidity during bloom has been more emphasized in the various studies. The yield is found not to be affected by chilling units but by number of cumulative hours with temperature between 14-20^0C and Relative Humidity 40-60% in case of Royal Delecious Apple.

Bud dormancy and temperature

Bud dormancy, break and development are affected by variation in temperature. Early bud break occurs when mean February temperature remains around 6.5^0C and delays when temperature falls below freezing point. High temperature ranging 18-21^0C during bud formation delays bud break. Insufficient chilling period during winter is responsible for delay in foliation. Besides, above ground temperature, variations in soil thermal regimes also impact on tree physiology. Root temperature around 20^0C is found to increase bud break over those at 10^0C. If the day time temperature is around 10^0C during pre bloom period, it may delay flowering in comparison to that of 15^0C.

Higher number of bud formation is favoured by long warm autumns with temperature more than 10^0C. Apple also require accumulated heat units for onset of flowering. It is found better to use daily temperature rather than using only cardinal temperature. Fruiting is also a temperature driven process. Temperature less than 4^0C and also more than 27^0C adversely affect bee activities besides the pollen germination. Temperature around 15^0C during full bloom increase fruit set. A sudden drop in temperature can kill the fruit lets. Night temperature above 16^0C around one month after full bloom can accelerate premature drops.

Cool nights favour the proper colour development due to increased anthocyanin synthesis, while, on the other hand, high temperature affect the same adversely. Flavour is also enhanced by low temperature at the time of harvest.

Incidence of diseases and insect-pest

Increased temperature is favoring the incidence of various diseases due to development of bacteria, viruses and fungus. In apple, already identified pathogens and pests cause damage to crop more frequently. This leads to increase in disease and pest incidence and climate change comprises shifting of disease ecology affecting apple production (Gautam et al., 2004). For this, control over pests and diseases require more frequent control measures. In this regard, number of pesticide sprays are increased from 4- 12 per year depending upon infestation.

Frost and freezing injury

There are two types of frosts that may affect the crop: wind frost and radiation frost. wind frost is not very much common in spring. Radiation frost occurs during clear sky nights. It can damage the full bloom stage up to 60-70% crop when temperature is around -2.2^0C. spring frost is not damaging as much as winter frost. A temperature of -3.5^0C at full bloom stage and -1.9^0C at bud closing can be critical for frost damage. Frost damage is less in late flowering cultivars.

A sudden drop in temperature to the tune of -10 to -15OC or a prolonged spells of -20OC can cause bud damage, besides damaging young and matured fruits subjected to the variation within cultivars. Freezing also badly hampers roots and bark woods. Roots are also adversely affected if temperature goes below -4^0C. Vertical cracks if the bark of stems also developed due to extreme fall in temperature.

Incidence of hailstorms

Hailstorms cause damage to flowers and developing fruits. Hailstorms result due to fluctuation in temperature in the atmosphere. This causes damage to the young plants. In apple orchards hail cause huge damage to young trees, cause flower drop and also damage to the developing fruits at various stages of development (Randev *et al.*, 2009).

Incidence of spring frost

Spring frost is resulted due to low temperature during growing time and this will cause frost injury to plant, flowers and fruit. In apple spring frost hampers pollination, damages young fruits hence resulting in poor fruit setting and yield loss.

Being a temperate crop, each phenophase of apple is directly affected by temperature to which it is exposed. It can be concluded that, in the changing climatic perspective, which mainly affect the air temperature, apple production in Uttrakhand will certainly going to be affected as in future.

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The second page should carry the full title of the manuscript and an abstract (of no more than 150 words for case reports, brief reports and 250 words for original articles). The abstract should be structured and state the Context (Background), Aims, Settings and Design, Methods and Materials, Statistical analysis used, Results and Conclusions. Below the abstract should provide 3 to 10 keywords.

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Standard journal article

[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006; 35: 540–7.

[2] Twetman S, Axelsson S, Dahlgren H, Holm AK, Källestål C, Lagerlöf F, et al. Caries-preventive effect of fluoride toothpaste: A systematic review. Acta Odontol Scand 2003; 61: 347–55.

Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone-iodine antisepsis. State of the art. Dermatology 1997; 195 Suppl 2: 3–9.

Corporate (collective) author

[4] American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. J Periodontol 2000; 71: 1792–801.

Unpublished article

[5] Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. Dent Mater 2006.

Personal author(s)

[6] Hosmer D, Lemeshow S. Applied logistic regression, 2nd edn. New York: Wiley-Interscience; 2000.

Chapter in book

[7] Nauntofte B, Tenovuo J, Lagerlöf F. Secretion and composition of saliva. In: Fejerskov O,

Kidd EAM, editors. Dental caries: The disease and its clinical management. Oxford: Blackwell Munksgaard; 2003. pp 7–27.

No author given

[8] World Health Organization. Oral health surveys - basic methods, 4th edn. Geneva: World Health Organization; 1997.

Reference from electronic media

[9] National Statistics Online—Trends in suicide by method in England and Wales, 1979–2001. www.statistics.gov.uk/downloads/theme_health/HSQ20.pdf (accessed Jan 24, 2005): 7–18. Only verified references against the original documents should be cited. Authors are responsible for the accuracy and completeness of their references and for correct text citation. The number of reference should be kept limited to 20 in case of major communications and 10 for short communications.

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