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BARLEY CHITINASE GENE CONFERS RESISTANCE AGAINST *FUSARIUM OXYSPORUM* AND *ALTERNARIA SOLANI* IN TRANSGENIC POTATO AK-22

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ABSTRACT

Fungal diseases are a constant threat to crop production worldwide. For chemical control of fungal diseases fungicides are used excessively which not only pollute the environment but also harm the human health. The increasing demand of production and regulations on the use of agrochemicals and the emergence of pathogens resistant to the products used, justifies the search for novel active molecules and new control strategies. Recent studies have shown that the transgenic approach is useful to control different fungal pathogens. A transgenic potato line was developed using barley *Chitinase* gene to control fungal pathogens. The transgenic potato AK-22 line was assessed under different biotic and abiotic factors i.e. fungal pathogens, saline environment and heavy metal stress. Herein, we tested the resistance of barley chitinase gene in potato to confers resistance against *Fusarium oxysporum* and *Alternaria solani* and found that the expression of barley chitinase gene enhanced with the time after infection in real time PCR amplification. The line found resistant against *Fusarium oxysporum, Alternaria solani* as well as tolerant against salinity and heavy metal stress.

Keywords: Fungal, Potato, Gene, Transgenic, Pathogens, Chemical, PCR

INTRODUCTION

Potato (*Solanum tuberosum* L.*)* is a worldwide crop, grown in different climatic conditions. However, moderate climate with a moderate temperature under long days gives the highest yield of potatoes. Potatoes are cultivated through an underground vegetative part known as tubers that increase the chance of disease transmission from one generation to another. Moreover, potatoes are susceptible to a wider variety of viruses, fungi, bacteria and insects, as these pathogens results in economic loss due to an attack of different diseases caused by them. Fungal diseases are the most destructive pathogen for potatoes, and about 70% of yield loss is reported in some parts of Pakistan due to a variety of fungal pathogens. In plants, pathogenic fungi are the major cause of yield reduction in economically important cash and food crops. Fungi like *Fusarium solani* and *Fusarium oxysporum* are the causative agents of wilting and damping off diseases in cotton, maize and rice. Similarly, *Alternaria alternata* causes leaf spots, thus reducing the yield of many cereal and food crops (Elhamid *et al*., 2010). There are different potato diseases in Pakistan which are black scurf, early blight, late blight, common scab, powdery scab, stem rot, soft rot, brown rot and other wilt diseases are generally serious fungal diseases of potatoes in Pakistan (Hussain *et al*., 2017). Plants being sessile, biotic and abiotic stress such as drought, temperature, heavy metals, pathogen interaction negatively influence the survival, biomass production and yield of potato plants (Agarwal *et al*., 2006). There are many heavy metals which pollute the soil. Theses metals are cadmium, salt, zinc, copper and nickel. These heavy metals only absorbed in the plant and not breakdown during normal metabolic process. Excess of heavy metals cause phytotoxic effect and eventually reduce the quality and yield of the crop (Gonalves *et al*., 2009). Fungi like *Fusarium oxysporum, Fusarium solani* and *Alternaria solani* cause diseases in potato (Al Mughrabi, 2010). *Fusarium oxysporum* also cause dry rot in different crops including potatoes and cause severe losses. *Fusarium* can not only destroy plant tissues but also produce toxins which are harmful to humans (Recep *et al*., 2009). Abiotic stress remains the greatest constrains to crop production (Jewel *et al*., 2010). Abiotic stress includes heavy metals stress (cadmium), drought, salinity, heat and cold stress. Abiotic stress greatly reduces the crop yield and plant growth (Verma, 2016). Potatoes are sensitive to salinity particularly in early stage. Potato yield decreases as the salinity increases in the soil or with irrigation water (Patell *et al*., 2001). Cadmium (Cd) is a non-essential element that negatively affects plant growth and development, Cd also reduces root growth in plants (Hasan *et al*., 2009). Low temperature leads to severe losses to potato crops (Parkin *et al*., 1989). Low temperature stress often occurs at 1 to 10ºC temperature, Low temperature has also effect on cellular metabolism (Morris *et al*., 1958). Different

approaches are used to control/prevent plant diseases, of which are chemical control, biological control and molecular approach (Pal and Spadden, 2006). Introduction of genes to the host that is resistant in some crops for specific pathogens to another crop to produce resistance are major techniques used in transgenic approach (Comelissen and Melchers, 1993). In 1942, discovery of DDT and lindane the first-generation synthetic insecticides give rise to a new era in the control of pests (Cabanilla, 2007). Numerous plant genes are used with varying degree of succession in crop improvements and host pathogen interaction (Gururani *et al*., 2012). Chitinases are enzymes that hydrolyze the N-acetyl glucosamine polymer chitin and they occur in diverse plant tissues over a broad range of crops (Punj and Zhang, 1993). Chitin a β-1,4-linked polymer of N-acetyl glucosamine, is a structural component of diverse array of organisms including fungi, bacteria and nematodes (Konagaya *et al*., 2006). The catabolism of chitin occurs typically in two steps involves the initial cleavage of chitin polymer by chitinase into chitin oligosaccharides, and then further cleavage to N-acetyl glucosamine monomers by chitobiasis (Suginta *et al*., 2000).

MATERIALS AND METHODS

Transgenic Potato plants with endo-chitinase gene were grown on MS (Murashige and Skoog) media in tissue culture tubes and place them in growth rooms. 21 days old plants were used in essays.

Preparation of spore suspension

Spore suspension of *Alternaria solani and Fusarium oxysporum* were prepared from fruit 7-10 days after inoculation, by gently brushing spores from the fruit with a small test-tube brush into about 500 ml of distilled water containing 0.05% of the wetting agent Lissapol LD®, (Lauryl alcohol sulphate in the presence of acetic anhydride).

Spraying

Plants were shifted to pots from tissue culture tubes. Place them in growth chamber for 3 days. Injured the plant with sterile carbon paper, spore suspension is sprayed on them and incubated at 25ºC with 36% humidity.

RNA Isolation

Sample from plants were taken at 0 hour (before spray) 7 days, 15 days, 21days and after a month of inoculation of pathogen. RNA extracted using TRIZOl® reagent and quantified by using NanoDrop™. Quantified RNA is then proceeding further to synthesis of complementary DNA. The Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit was used which allows for total RNAs to be converted to cDNA through ProFlex PCR thermal cycler. The targeted mRNA primers were then used for qRT PCR protocols. The SYBR assay of real time PCR by using Maxima SYBR Green/ROX qPCR Master Mix (2X) was performed using a three-step protocol in AriaMX Real – Time PCR System (Agilent Technologies) instrument.

Statistical analysis: Statistical analysis of the data was conducted using SPSS and Microsoft Excel.

RESULTS

Biotic stress essays

After spraying, the data was recorded for a month to evaluate results. Disease chart for *Alternaria solani* is in Table 1 and disease chart for *Fusarium oxysporum* is in Table 2 and significant differences were shown between control and AK-22 (Fig 1 and Fig 2)

Table 1. Disease chart for *Alternaria solani.*

	Control	Ak-22 D	AK-22 E	AK-22 F
Number of leaves	7	15	21 in 2 branches	18
Plant length before inoculation (cm)	8.3	7	9	8
Plant length after inoculation (1month) (cm)	8.3	15.8	14.7	15.5
Diseased leaves	22	25	25	22
Number of spots per leaf	5	4	4	5
Leaves affected first	Older leaves Lower leaves	Older leaves Lower leaves	Older leaves Lower leaves	Older leaves Lower leaves
Other symptoms	Stem thickening Yellowing, mild wilting	No other symptoms	Affected leaves yellow	Affected leaves yellow
Temperature	26.7ºC	26.7ºC	26.7ºC	26.7ºC
Humidity	35%	35%	35%	35%

Table 2. Disease chart for *Fusarium oxysporum.*

	Control	AK-22 A	AK-22 B	AK-22 C
Number of leaves	13	14	15	13
Plant length before inoculation(cm)	17.9	17.1	17.8	18.86
Plant length after inoculation (1month) (cm)	17.9	27.9	25.4	25.4
Diseased leaves	12	2	3	2
Number of spots per leaf	8	6	5	4
Leaves affected first	Older leaves Lower leaves	Older leaves Lower leaves	Older leaves Lower leaves	Older leaves Lower leaves
Other symptoms	Wilting, yellowed then died	No wilting except affected leaves	No wilting except affected leaves	No wilting except affected leaves
Temperature	26.7ºC	26.7ºC	26.7ºC	26.7ºC
Humidity	35%	35%	35%	35%

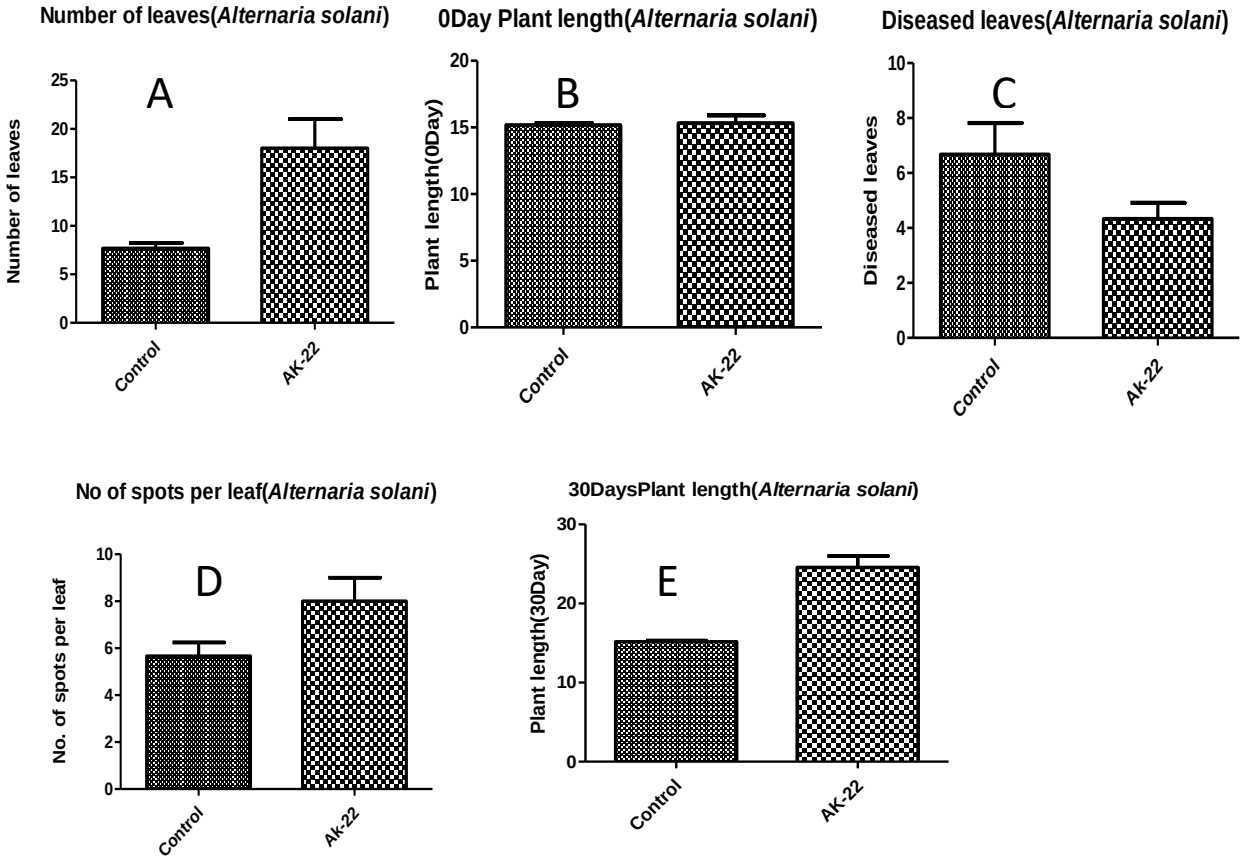

Fig. 1. A and B revealed non-significant difference between control and Ak-22 (P<0.05). C, D and E show the significant difference between control and AK-22 (P<0.05). The coefficient of variance was less than 20% in all results which indicated the consistency of results.

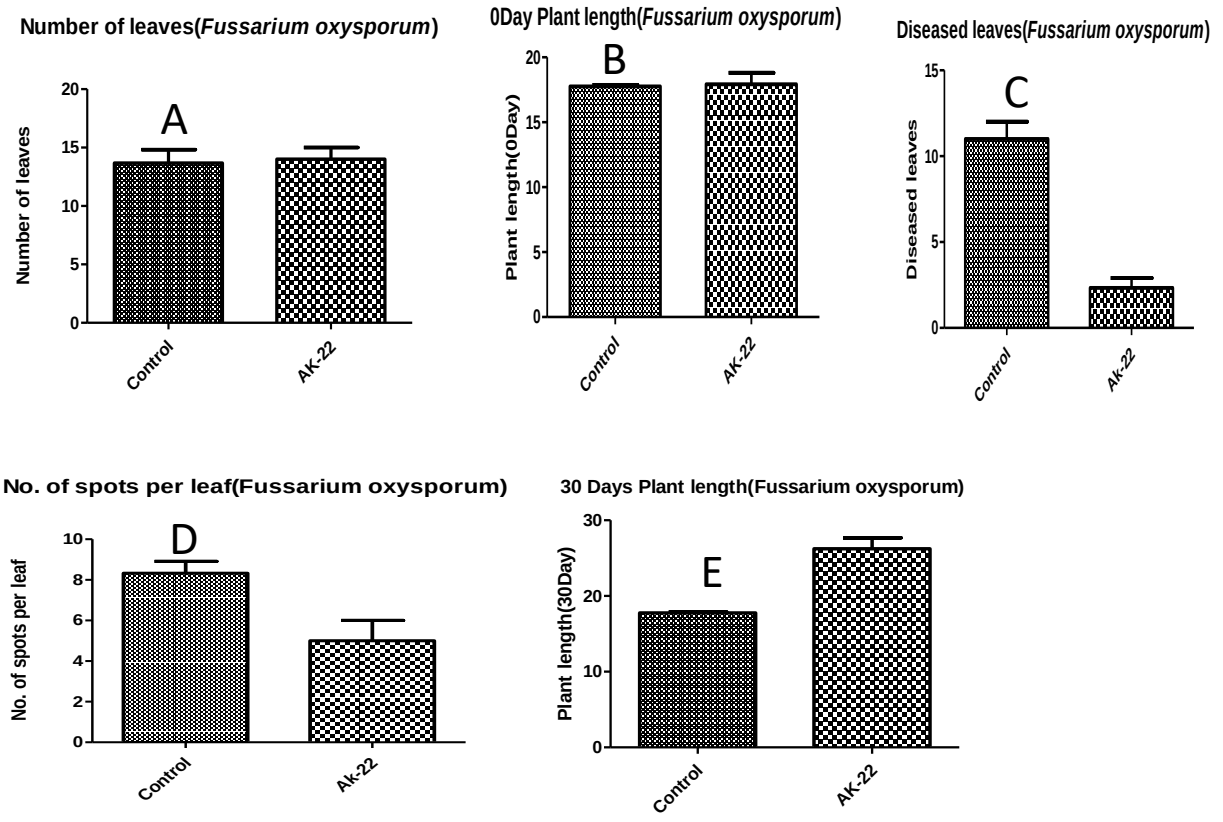

Fig. 2. A and B revealed non-significant difference between control and Ak-22 (P<0.05).C, D and E show the significant difference between control and AK-22 (P<0.05). The coefficient of variance was less than 20% in all results which indicated the consistency of results.

In-vitro antifungal essay

In-vitro antifungal essays were performed shows that the transgene performed well and grows in fungus for more than 20 days. Control plants were yellowed first and died (Fig 3).

Fig. 3. Transgenic AK-22 plants shows resistance invitro against fungi

Chitinase activity essay

RNA of transgenic plant having *endochitinase* gene (ChiB) was isolated using standard TRIZOL® method at various time intervals 0 h, 6 h, 12 h, 24 h, 36 h, 15 days and 1 month. cDNA synthesized and chitinase activity was observed using *chitinase* specific primers in RT-PCR with SYBR Green dye. Percentage Knock Down value was calculated to measure chitinase activity level (Table 3).

Table 3. Calculation of Percent Knock Down Value of Chitinase Activity.

	Cq	Cq	ΔCq	ΔCq Expression	Mean ΔCq	ΔΔCq	% KD
Time	Control	Treatment			Normalized		
0H	18.09	20.18	2.09	0.234881	0.405189		
	19.99	21.18	1.19	0.438303			
	18.45	19.08	0.63	0.646176			
6H	16.93	18.26	1.33	0.397768	0.243164	0.600124	99.8216
	16.18	18.36	2.18	0.220676			
	15.9	18.51	2.61	0.163799			
12H	12.27	13.45	1.18	0.441351	0.250578	0.618423	98.2313
	12.51	14.55	2.04	0.243164			
	11.77	14.54	2.77	0.146604			
24H	12.55	14.11	1.56	0.339151	0.268563	0.66281	97.6562
	12.98	15.01	2.03	0.244855			
	12.22	14.32	2.1	0.233258			
36H	13.67	15.69	2.02	0.246558	0.274841	0.678302	95.8684
	12.28	14.11	1.83	0.281265			
	13.37	15.11	1.74	0.29937			
15Days	12.56	14.45	1.89	0.269807	0.284533	0.702222	95.3107
	13.67	15.13	1.46	0.363493			
	12.78	14.87	2.09	0.234881			
1 Month	20.22	22.1	1.88	0.271684	0.329877	0.814131	94.5053
	21.33	23.18	1.85	0.277392			
	21.01	22.08	1.07	0.476319			

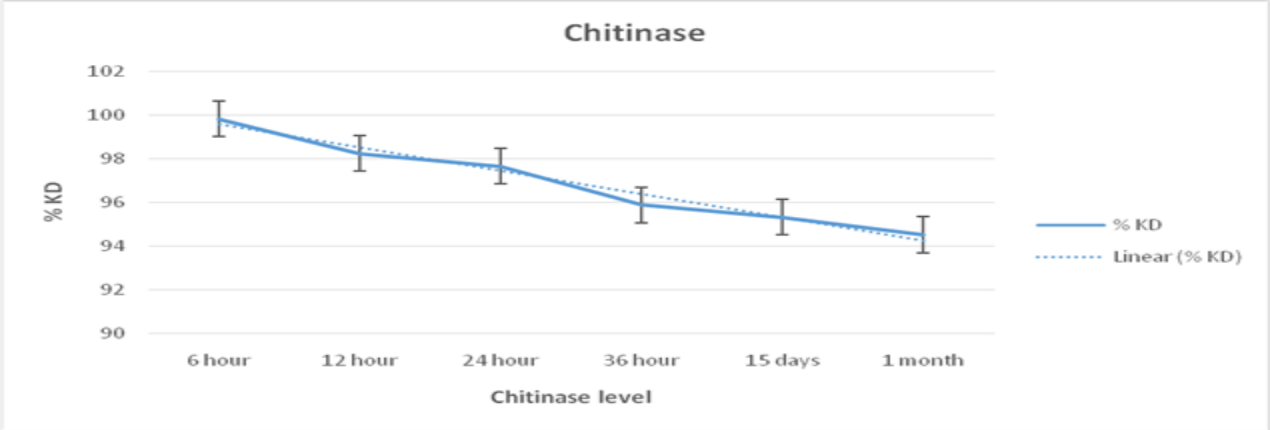

Fig. 4. Represents chitinase level. Increase in percent KnockDown (%KD) value means decrease in expression of chitinase gene and decrease in %KD value means increase in expression of chitinase gene. At this stage the firstly detected the chitinase gene but within time, gene level was increased.

As the graph indicates at 6 hours of inoculation, the chitinase gene starts expressing and as time passes to 1 month, the chitinase gene expression was maximum (Fig 4).

DISCUSSION

Plant diseases are of major concern in the agriculture as they cause huge losses both in industry and economy of the nation. Abiotic stress also contributed in the yield loss annually. Plant protection in general and the protection of crops against diseases, have an obvious role to play in meeting the growing demand for food quality and quantity (Savary *et al*., 2012). Among all losses, major losses are caused by fungi, almost all crop plants are attacked by pathogenic fungi. Many chemical, physical and biological ways are used to overcome fungal pathogen to control diseases. Excessive use of fungicides not only infect crop but also harm the human health (Dellavalle *et al*., 2011). Biological control is the use of natural microorganisms to control other pathogens, a practice widely used now days instead of fungicides to protect environment as well as soil environment (Gerbore *et al*., 2014). Despite pathogenic losses, huge losses are caused by different environmental and soil factors includes salinity, alkalinity and heavy metals. The increasing demand of production and regulations on the use of agrochemicals and the emergence of pathogens resistant to the products employed, justifies the search for novel active molecules and new control strategies.

Potato production has been expanded in recent times and *Solanum tuberosum* is now one of the five most important food crops. Potatoes produce more starch per hectare than any other crop (Cabanilla *et al*., 2007). Recent studies have shown that the transgenic approach is useful in control of different pathogens and different stress conditions. Potato line is developed with a barley chitinase gene to overcome pathogenic and abiotic stress conditions.

In recent studies, the transgenic potato AK-22 lines are accessed by different factors include biotic i.e. fungal pathogens and abiotic i.e. saline and heavy metal stress. Fungi include in the experiment are *Fusarium oxysporum* and *Alternaria solani* with various methods to check pathogenicity and activity of chitinase gene in accordance with the resistance induce by chitinase gene. These fungi are tested by spore spray and in-vitro antifungal analysis. Abiotic stress also studied includes saline stress, heavy metals stress and low temperature stress with different concentrations of salts.

CONCLUSION

It is concluded that the results of desired chitinase gene performed very well in the essays. The transgenic plant AK-22 is resistant to many fungal pathogens under stress conditions. It may be more useful for controlling of various fungal diseases and can be utilized for controlling of various pathogens in various crop species under different environmental conditions. The study is equally beneficial for scientists, researchers and farmers community to start up a new research program to develop new crop varieties by utilization of such types of techniques.

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REFERENCES

Agarwal, P.K., P. Agarwal, M.K. Reddy and S.K. Sopory (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports*, 25(12): 1263-1274.

Al-Mughrabi, K. I. (2010). Biological control of Fusarium dry rot and other potato tuber diseases using Pseudomonas fluorescens and Enterobacter cloacae. *Biological Control*, 53(3): 280-284.

Cabanilla, L.S. (2007). Socio-economic and political concerns for GM foods and biotechnology adoption in the Philippines. *AgBioForum*, 10(3):178-183.

Cornelissen, B.J.C. and L.S. Melchers (1993). Strategies for Control of Fungal Diseases with Transgenic Plants. *Plant Physiology*, 101(3): 709-712.

Dellavalle, P.D., A. Cabrera, D. Alem, P. Larrañaga, F. Ferreira and M.D. Rizza (2011). Antifungal activity of medicinal plants extracts against phytopathogenic fungus Alternaria spp. *Chilean Journal of Agricultural Research*, 71: 231-239.

Elhamid, M., H. Makboul, M. Sedik, I. Ismail and M. Ibrahim (2010). Cloning, expression and antifungal acti vity of an endochitinase gene derived from barley (Hordeum Vulgare), *Research Journal of Agriculture and*

Biological Sciences, 6(3): 356-363.

Gerbore, J., J. Vallance, A. Yacoub, F. Delmotte, D. Grizard, C. Regnault-Roger and P. Rey (2014). Characterization of Pythium oligandrum populations that colonize the rhizosphere of vines from the Bordeaux region. *FEMS Microbiology Ecology*, 90(1): 153-167.

Gonalves, J.F., A.G. Becker, L.B. Pereira, J.B.T. da Rocha, D. Cargnelutti, L.A. Tabaldi, V. Battisti, J.G. Farias, A.M. Fiorenza, M.M. Flores, F.T. Nicoloso and MRC. Schetinger (2009). Response of cucumis sativus L. seedlings to Pb exposure. *Brazilian J Plant Physio*, 21(3): 175-186.

Gururani, M.A., J. Venkatesh, C.P. Upadhyaya, A. Nookaraju, S.K. Pandey and S.W. Park (2012). Plant disease resistance genes: Current status and future directions, *Physiological and Molecular Plant Pathology*, 78: 51-65.

Hasan, S.A., Q. Fariduddin, B Ali, S. Hayat and A. Ahmad (2009). Cadmium: Toxicity and tolerance in plants. *Journal of Environmental Biology March J. Environ. Biol*, 30(2): 165-174.

Hussain, A., S.W. Khan, M.S. Awan, S. Ali, Q. Abbas, Z. Ali and S. Ali (2017). Potato black scurf , production practices and fungitoxic efficacy of rhizoctonia solani isolates in hilly areas of gilgit-baltistan pakistan. *Pak J Bot*, 49(4): 1553-1560.

Jewell, M.C., B.C. Campbell and I.D. Godwin (2010). *Transgenic Plants for Abiotic Stress Resistance*. Heidelberg, Germany; Springer Verlag, Berlin, Heidelberg, 67-132.

Konagaya, Y., C. Tsuchiya and H. Sugita (2006). Purification and characterization of chitinases from Clostridium sp. E-16 isolated from the intestinal tract of the South American sea lion (Otaria flavescens). *Letters in Applied Microbiology*, 43(2): 187-193.

Morris, L., C.C. Charles, W.V. Audia and M.S. Wilcox (1958). Biochemical Studies of Chilling Injury in Sweet Potato. *Plant Physiology*, 33(5): 307-311.

Pal, K.K. and G.B. Spadden (2006). Biological Control of Plant Pathogens. *The Plant Health Instructor*, 1–25.

Parkin, K.L., A. Marangoni, R.L. Jackman, R.Y. Yada and D.W. Stanley (1989). Chilling Injury. a Review of Possible Mechanisms. *Journal of Food Biochemistry* 13(2): 127–153.

Patell, R.M., S.O. Prasher, D. Donnelly and R.B. Bonnell (2001). Effect of initial soil salinity and subirrigation water salinity on potato tuber yield and size. *Agricultural Water Management*, 46(3): 231–239.

Punj, Z.K. and Y.Y. Zhang (1993). Plant Chitinases and Their Roles in Resistance To Fungal Diseases, *Journal of Nematology*, 25(4): 526-540.

Recep, K., S. Fikrettin, D. Erkol and E. Cafer (2009). Biological control of the potato dry rot caused by Fusarium species using PGPR strains. *Biological Control*, 52: 194-198.

Savary, S., A. Ficke, J.N. Aubertot and C. Hollier (2012). Crop losses due to diseases and their implications for global food production losses and food security. *Food Security*, 4(4): 519-537.

Suginta, W., P.A.W. Robertson, B. Austin, S.C. Fry and L.A. Fothergill-Gilmore (2000). Chitinases from Vibrio: Activity screening and purification of chiA from Vibrio carchariae. *Journal of Applied Microbiology*, 89(1): 76-84.

Verma, S.D. (2016). Abiotic Stress and Crop Improvement: Current Scenario. *Advances in Plants & Agriculture Research*, 4(4): 1–2.

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