Content list available at http://epubs.icar.org.in, www.kiran.nic.in; ISSN: 0970-6429

Indian Journal of Hill Farming

December 2018, Volume 31, Issue 2, Page 323-328

Effect of Different Chemical Compounds and Nano-Formulations on the Cell Wall Components of *Alternaria solani* and *A. alternata*

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ARTICLE INFO

Article history:
Received 28 February 2018
Revision Received 1 September 2018
Accepted 19 October 2018

Key words:
Nano-particles, Conventional chemicals, Alternaria, Glucose, Protein, Lipid

ABSTRACT

The effect of certain chemical compounds, *i.e.* chitosan nano-particle, silver nitrate, silver nano-particle, silvox, salicylic acid, hydrogen peroxide, titanium, titanium dioxide hydrophobic and hydrophillic nano-particle, silicon, silicon dioxide hydrophilic and lipophillic nano-particle in comparison with isoprothiolane (check fungicide), was assessed at 20 ppm on the glucose, protein, lipid contents of the mycelium of *Alternaria solani* and *A. alternata* which cause tremendous loses in potato cultivation through early blight. The isolation and identification of the pathogens, harvest of its mycelium and analysis was done following standard protocols. There was a decrease in the level of glucose and protein and increase of lipid in the mycelium using the compounds. Nano-formulations were effective over conventional chemicals including check fungicide. Silver nano-particle was most effective amongst the nano-formulations and silver nitrate amongst the conventional chemicals. The efficacy of remaining compounds was better than check fungicide. From this, it was concluded that nano-particles and conventional chemical compounds can be used to replace synthetic fungicides to control the attacks of early blight through altering the cell wall components of the causative agents.

1. Introduction

The global food production suffers most due to various plant diseases and millions of dollars are being spent every year against these diseases. Fungal diseases are of greatest concern among these diseases because of its intensity and severity. Recently development of safe management which pose less danger to the humans and animals have been emphasized (Ouda 2014). Various natural and artificial methods are recommended and applied against these diseases; amongst pesticide use is the most prevalent. But, environmental hazards due to use of pesticides have been widely criticized in the recent era and thus research diverted towards alternative and eco-friendly measures. In this direction, use of nano-particles and conventional chemical compounds which are already exist in the host- defense mechanisms of the plant, are becoming popular (Jo et al. 2009) over synthetic pesticides because of their efficacy and environmental-safety. Nano-formulations have greater adhere ability to the microbes and degrading its multiplication ability (Derbalah et al. 2013). There are compounds of silver, chitosan, silicon, titanium origin which are effective against various plant pathogens including *Alternaria* (Bhattacharjee et al. 2017), however, study on the effect of these compounds on the cell compounds of the fungi are greatly lacking. In this study, certain of these chemicals were evaluated on cell wall components, *i.e.* glucose, protein and lipid of *Alternaria solani and A. alternata* of potato origin so that strategies could be formulated against the huge losses incurred in food crops due to them (Pandey and Vishwakarma 1998).

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2. Materials and Methods

The study was conducted in the Department of Plant Pathology of Bidhan Chandra Krishi Viswavidyalaya (BCKV), West Bengal. For routine phytopathological and analytical techniques, standard methods have been followed (Bhattacharjee et al. 2016a, b). The pathogens, *i.e. A. solani* and *A. alternata* were isolated from the infected potato leaves collected from major potato growing areas of West Bengal. The morphological and cultural characteristics of the isolated fungi were confirmed through observation of greyish brown colonies, muriform, light-brown and long-beaked conidia in *A. solani* and short-beaked in *A. alternata* (Chowdhry et al. 2000). To see the effect of chemical compounds namely- silver nitrate ($AgNO_3$; Merck India), silver nano-particle (AgNP; Source- Assam Agricultural University, Jorhat), silvox (H_2O_2 and AgNP; Source- Chemtex speciality Limited, Kolkata, India), chitosan nano-particle (CNP; Source- Assam Agricultural University, Jorhat), hydrogen peroxide (H_2O_2; Merck India), salicylic acid (SA; Rankem), isoprothiolane (40% EC, Check fungicide-CF; Parijat industries (INDIA) Pvt. Ltd.), chitosan (BCKV, West Bengal), titanium dioxide hydrophilic nano-particle (TiO_2- hpl NP; Source-ISI, Kolkata), titanium dioxide hydrophobic nano-particle (TiO_2- hpb NP; Source-ISI, Kolkata), silicon dioxide hydrophobic nano-particle (SiO_2-HNP; Source-ISI, Kolkata), silicon dioxide lipophilic nano-particle (SiO_2-LNP; Source-ISI, Kolkata), silicon (Source-BCKV, West Bengal) and titanium (Source-BCKV, West Bengal), the fungi were grown in potato-dextrose agar (PDA) with the chemicals at 20 ppm. The dose was set at 20 ppm being it produced maximum inhibition during i*n-vitro* trials through the poisoned food technique (Nene and Thapliyal 1979). In control plate, distilled water used instead of chemicals. For the analysis of cell wall components, fungal mycelium was harvested from the culture plate by filtering (Whatman No. 1) after 48 h of growth. The mycelial pellet was washed thoroughly with distilled water and then used for glucose, protein and lipid content.

Glucose was estimated by the GOD/POD method using *in vitro* diagnostic kits (Siemens Ltd., Gujarat). Briefly, three test tubes were marked as blank, standard and test. Then one ml of working solution was poured to all the three test tube. 10 µl of standard solution was added to the test tube marked as 'standard', 10 µl of sample was added to the test tube and marked as 'test'. Then the test tubes were incubated at 37^0C for 15 minutes and mixed thoroughly. The absorbance was read at 505 nm in a UV Spectrophotometer (Thermo) and the glucose level was determined using the formula, glucose (mg/ml) = {(Absorbance of sample/Absorbance of standard) x Conc. of standard}. A portion of the pellet was macerated in an Eppendorf tube using known volume of distilled water and centrifuged at 10000 rpm for 10 min at 4^0C. Then supernatant was collected for protein estimation through Biuret method using *in vitro* diagnostic kit (RFCL Ltd., Uttarakhand). Briefly, three clean test tubes were taken and marked as blank, standard, and test. Then one ml working reagent was added to all test tubes. Then 20 µl of calibrator was added to the test tube marked as 'Standard', 20 µl of sample was added to the tube marked as 'Test' and 20 µl of distilled water was added to the test tube marked as 'Blank'. The solutions were mixed well and allowed to stand at room temperature for 20 minutes. Then the absorbance was read at 546 nm in a UV Spectrophotometer (Thermo) and protein level was determined using the formula, total protein (mg/ml) = {(Absorbance of unknown /Absorbance of calibrator) X calibrator value}. The lipid content was estimated through solvent extraction methods. Briefly, 0.20 g of mycelial pellet was weighed into an extraction thimble and covered with cotton. Then 50 ml petroleum ether was added to a pre-weighed cup. Both thimble and cup were attached to the extraction unit. The sample was subjected to extraction with solvent for 30 min followed by rinsing for one and half hour. The solvent was evaporated from the cup to the condensing column. Extracted lipid in the cup was placed in an oven at 110^0C for an hour and after cooling, the crude lipid content was calculated using formula, Crude lipid (mg/ml) = (Extracted lipid/ sample weight) X 100. The data were analysed in SPSS (Ver. 21) using one-way analysis of variance (ANOVA) and expressed as mean±S.E. The significant difference among the means of different components was compared with Duncan's Multiple Range Test at 95% level of significance.

3. Result and Discussion

The glucose, protein and lipid contents of *A. solani* and *A. alternata* in response to the chemical compounds including nano-formulations are presented in Table 1 and 2 which showed the decreased level of glucose and protein and increased level of lipid in the mycelium of the fungi (Ouda 2014). Nano-formulations were effective over conventional chemicals including CF due to its higher adherence to the cell surface of fungi, degrading its lipopolysaccharide molecules and increasing the permeability of cell membrane (Derbalah et al. 2013), which however need further understanding (Hwang et al. 2008). Among the nano-formulations, AgNP followed by CNP, SiO_2-HNP, SiO_2-LNP, TiO_2- hpb NP, TiO_2- hpl NP and among the conventional chemicals, $AgNO_3$ followed by Silvox, SA, H_2O_2, chitosan, Si and Ti were effective over CF.

Table 1. Effect of different chemical compounds on the glucose, protein and lipid contents of mycelium of *Alternaria solani*

Compounds	Glucose (mg/ml)	Protein (mg/ml)	Lipid (mg/ml)
Control	262.33±13.09^{f}	17.57±1.04^{d}	5.87±0.18^{a}
CF	209.67±20.99^{e}	13.40±2.32^{d}	8.66±1.15^{b}
$AgNO_3$	188.33±10.37^{de}	9.25±1.22^{bc}	8.76±0.18^{b}
Silvox	189.33±12.72b^{de}	9.28±0.75^{bc}	8.86±0.34^{b}
SA	189.67±11.14^{de}	9.30±1.37^{bc}	9.10±0.26^{b}
H_2O_2	192.25±8.63^{de}	9.73±1.65^{c}	9.13±0.29^{b}
Chitosan	194.33±8.84^{de}	10.17±0.71^{c}	9.22±0.54^{b}
Si	194.67±12.06^{de}	10.30±1.16^{c}	9.24±0.85^{b}
Ti	205.52±18.26^{de}	12.84±1.85^{d}	9.30±0.49^{b}
AgNP	132.33±12.03^{a}	5.93±0.30^{a}	12.73±0.39^{d}
CNP	145.43±12.12^{ab}	6.15±0.43^{a}	11.86±0.44^{d}
SiO_2-HNP	162.24±6.56^{b}	6.33±0.94^{a}	10.57±0.91^{e}
SiO_2-LNP	165.00±6.81^{bc}	7.11±0.85^{a}	9.83±0.54^{bc}
TiO_2- hpb NP	185.23±13.12^{cd}	8.68±1.65^{bc}	9.70±0.49^{bc}
TiO_2- hpl NP	187.24±11.24^{de}	9.12±1.25^{bc}	9.60±1.65^{bc}

This varying intensity of effect was dependent upon the size, core composition, shape, surface property, purity, stability, method of synthesis and reactivity of the particles from compound to compound (Teske and Detweiler 2015). Glucose reduction in *A. solani* was 29.6% higher with AgNP, 24.6% higher with CNP, 18.2% higher with SiO_2-HNP, 17.1% higher with SiO_2-LNP, 10% higher with TiO_2-hpb NP, 8.6% higher with TiO_2-hpl NP, 8% higher with $AgNO_3$, 7.8% higher with silvox, 7.7% higher with SA, 6.7% higher with H_2O_2, 6% higher with chitosan, 5.8% higher with Si and 1.6% higher with Ti when compared with CF. It was significant ($p \leq 0.05$) in AgNP, CNP, SiO_2-HNP, SiO_2-LNP and TiO_2-hpb NP, but insignificant ($p \geq 0.05$) in other compounds when compared with CF (Table 1). In *A. alternata*, glucose reduction was 25.8% higher with AgNPs, 20.6% higher with CNP, 16% higher with SiO_2-HNP, 15.9% higher with SiO_2-LNP, 14.8% higher with TiO_2-hpb NP, 12.9% higher with TiO_2-hpl NP, 12.7% higher with $AgNO_3$, 11% higher with silvox, 10.6% higher with SA, 9.3% higher with H_2O_2, 8.8% higher with chitosan, 8.8% higher with Si and 6.6% higher with Ti and it was significant ($p \leq 0.05$) in all compounds when compared with CF except Ti (Table 2). This reduction of glucose could be attributed to the interference of the chemicals with normal functioning of respiratory sugar, and enzymes of the fungi responsible for ATP production and energy balance, formation of phytoalexins, lignin and callose, inhibition of catalase activity *etc.* (Yamanaka et al. 2005). Bhattacharjee et al. (2017) also reported the inhibition of mycelial growth of *Alternaria* due to same compounds. Protein reduction in *A. solani* was 42.8% higher with AgNP, 41.5% higher with CNP, 40.5% higher with SiO_2-HNP, 36.5% higher with SiO_2-LNP, 27.5% higher with TiO_2-hpb NP, 24.5% higher with TiO_2-hpl NP, 23.9% higher with $AgNO_3$, 23.7% higher with silvox, 23% higher with SA, 21.1% higher with H_2O_2, 18.6% higher with chitosan, 17.9% higher with Si and 3.5% higher with Ti and it was significant ($p \leq 0.05$) when compared with CF (Table 1). In *A. alternata*, protein reduction was 45% higher with AgNP, 33.2% higher with CNP, 33% higher with SiO_2-HNP, 32.3% higher with SiO_2-LNP, 30.8% higher with TiO_2-hpb-NP, 27.1% higher with TiO_2-hpl-NP, 27.1% higher with $AgNO_3$, 22.3% higher with silvox, 20.9% higher with SA, 20.2% higher with H_2O_2, 18.2% higher with chitosan, 13.9% higher with Si and 9.6% higher with Ti and significant ($p \leq 0.05$) when compared with CF (Table 2). This decrease of protein could be due to the adherence of the chemicals with cell wall of the fungi and denaturation of proteins, inhibition of DNA replication (Feng et al. 2000) and mRNA synthesis (Sudarshan et al. 1992), inactivation of the expression of ribosomal subunit proteins, production of reactive oxygen species (ROS) and increasing the permeability of cell membrane and leakage of cellular contents, accumulation of proteinase inhibitors *etc.* Similar effect was reported in other fungi due to silver (Kim et al. 2012), chitosan (Kong et al. 2010; Ahmed 2017) and titanium dioxide (Suriyaprabha et al. 2014). The lipid increase in *A. solani* was 63.9% higher with AgNP, 54.5% higher with CNP, 32.5% higher with SiO_2-HNP, 20% higher with SiO_2-LNP, 17.8% higher with TiO_2- hpb-NP, 16% higher with TiO_2-hpl NP, 1.7% higher with $AgNO_3$, 3.5% higher with silvox, 7.5% higher with SA, 8% higher with H_2O_2, 9.5% higher with chitosan, 9.9% higher with Si and 10.9% higher with Ti and significant ($p \leq 0.05$) when compared with CF (Table 1). In *A. alternata,* lipid increase was 78.2% higher with AgNP, 66% higher with CNP, 57.2% higher with SiO_2-HNP, 44.8% higher with

Table 2. Effect of different chemical compounds on the glucose, protein and lipid contents of mycelium of *Alternaria alternata*

Compounds	Glucose (mg/ml)	Protein (mg/ml)	Lipid (mg/ml)
Control	291.33±11.39^{f}	15.60±1.15^{f}	6.47±0.81^{a}
CF	221.00±14.15^{e}	13.23±1.70^{e}	7.67±1.79^{ab}
$AgNO_3$	184.23±5.65^{cd}	9.00±2.33^{bc}	9.43±3.43^{abcd}
Silvox	189.09±7.07^{cd}	9.74±0.70^{bcd}	9.20±3.01^{abcd}
SA	190.33±17.57^{cd}	9.97± 0.40^{bcd}	9.13±1.06^{abcd}
H_2O_2	194.33±4.63^{cd}	10.07±1.50^{bcd}	8.97±0.47^{abcd}
Chitosan	195.67±7.45^{cd}	10.70±1.80^{bcd}	8.57±2.23^{abc}
Si	195.71±13.01^{cd}	11.06±1.12^{cde}	8.11±0.09^{abc}
Ti	202.01±10.01^{de}	11.73±1.37^{de}	7.89±1.98^{ab}
AgNP	146.00±11.59^{a}	6.20±0.40^{a}	12.73±0.68^{e}
CNP	161.33±15.01^{ab}	8.05±0.09^{ab}	11.94±0.01^{de}
SiO_2-HNP	174.51±17.54^{cd}	8.08±1.50^{ab}	11.37±1.50^{cde}
SiO_2-LNP	175.06±11.01^{cd}	8.19±1.90^{ab}	10.57±1.23^{bcde}
TiO_2- hpb NP	178.33±11.86^{ab}	8.43±1.16^{ab}	10.13 ±0.50^{bcde}
TiO_2- hpl NP	183.66±12.02^{cd}	9.0±1.37^{bc}	9.87±2.08^{bcde}

SiO_2-LNP, 38% higher with TiO_2-hpb NP, 34% higher with TiO_2-hpl NP, 27.2% higher with $AgNO_3$, 23.7% higher with silvox, 22.6% higher with SA, 20.1% higher with H_2O_2, 13.9% higher with chitosan, 6.8% higher with Si and 3.5% higher with Ti, however, and it was insignificant ($p \geq 0.05$) in conventional compounds when compared with CF (Table 2). Lipid increase was a sign of oleaginous nature of the fungi which accumulated intracellular lipid under stress condition created due to interaction of the chemicals with negatively charged phospholipids of fungi membrane (Mukhopadhyay et al. 2011). This could also be due to excess of carbon availability to the cells during the period when other nutrients like protein, glucose *etc.* which are required for cell proliferation were exhausted from the system which however, deserves further study. Choudhury et al. (2012) also reported unusual accumulation of saturated fatty acids in *Aspergilus* in response to chemical compounds. Thus, the study concluded that conventional chemical compounds including nano-particles have significant effect on the cell wall components of *A. solani* and *A. alternata* through decreasing glucose and protein level and increasing lipid level of mycelium. Nano-formulations are more effective than conventional chemicals including check fungicide. Amongst the nano-formulations evaluated, silver nano-particle is best and amongst conventional chemicals, silver nitrate is best. The information can be used to formulate strategies against the infections due to *A. solani* and *A. alternata* in potato and to replace synthetic pesticides more effectively. Further study is required to assess the effect of these compounds on other plant pathogenic fungi for sustainable agriculture.

Acknowledgements

The authors are thankful to the Vice-Chancellor of the Bidhan Chandra Krishi Viswavidyalaya, West Bengal for extending all facilities to conduct this study.

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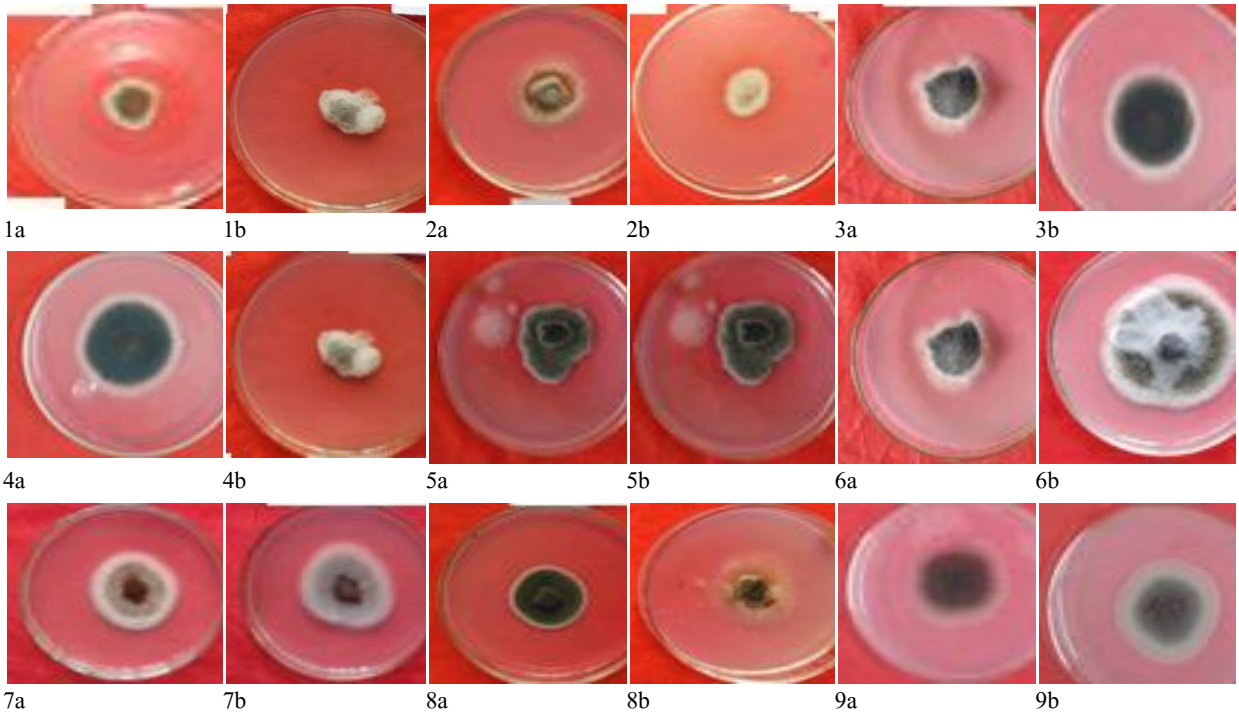

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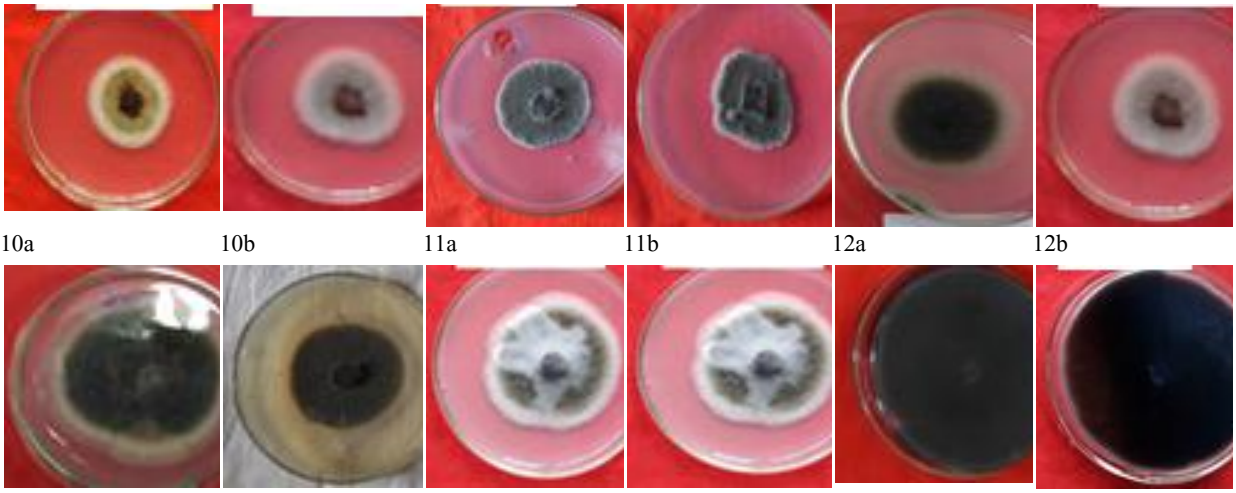

Plate 1. a- *A. solani* b-*A. alternata;* 1-AgNP; 2-CNP; 3-SiO_2-HNP; 4- SiO_2-LNP; 5- TiO_2-hpb-NP; 6- TiO_2-hpl-NP; 7-$AgNO_3$; 8-silvox; 9-SA; 10-H_2O_2; 11-chitosan; 12-CF; 13-Ti; 14-Si; 15-control