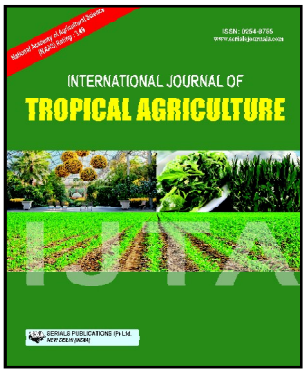

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Effect of different nitrogen sources, water and pH in apple pomace medium for spore production of biocontrol agent, *Trichoderma harzianum*

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Abstract: Apple pomace is standardized by supplementing the different nitrogen sources viz., ammonium sulphate $(NH4)_2SO4$, sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), water and pH for growth and spore production of *Trichoderma harzianum* in laboratory conditions. Results showed that among different concentrations evaluated, addition of 0.5 g $(NH4)_2 SO_4$ (10.82 ± 0.35 crore spores/ml), 1.0 g $NaNO_3$ (14.80 ± 0.42 crore spores/ml), 1g KNO_3 (10.45 ± 0.16 crore spores/ml), 4g NH_4NO_3 (13.17 ± 0.31 crore spores/ml), 50 ml water (9.65 ± 0.20 crore spores/ml) and pH 9 (10.53 ± 0.08 crore spores/ml) showed higher spore production of *T. harzianum.*

Key words: Apple pomace, *Trichoderma harzianum*, nitrogen sources, water, pH

INTRODUCTION

The increased use of conventional chemical pesticides over the years has not only contributed to an increase in food production, but also resulted in adverse effects on the environment and non-target organisms. Due to indiscriminate use of pesticides for the control of pests and diseases which led to development of resistance, persistence and pesticide residues. The application synthetic fungicides directly to the soil for the control of soil borne pathogens which affects beneficial soil microorganisms, fauna and soil fertility. Use of bio-pesticides for the control of pests and diseases has increased the global attention[1-5]. In India few commercial bio-pesticides are available in the market and lacks quality aspects (virulence, spore load, pathogenicity, viability etc.) as compared to other countries. At present farmers are applying synthetic fungicides extensively

for the control of soil-borne pathogens as compared to biofungicides due to non-availability and lack of quality. In India, all commercial bio-pesticide producers using liquid media for the large scale production of blastospores for commercial formulation due to un availability of agricultural waste. The main issues involved for the mass production of *Trichodema* sp., is the development of cost-effective methods. In India growers/farmers are cultivating vegetables (cabbage, cauliflower, peas, potato, tomato etc.) and flower crops (gerbera, carnation, gladiolus etc.,) under field and greenhouse conditions. Among pests and diseases reported on these crops, the soil borne pathogens viz., *Pythium*, *Phytophthora, Rhizoctonia*, *Fusarium*, *Verticillium* etc., are major problems under open and protected cultivation due to mono-cropping.

Apple (*Mallus domestica* Borkh) is most important fruit crop widely grown in temperate regions of the world including India. In the world, apple grown in 5.05 million hectare with a production of 84.63 million tonnes in 2014. In India the apple is grown an area of 2.77 lakh hectares with annual production of 25.20 lakh tonnes [6]. Apple pomace (AP) is left over waste after extraction of juice which contains peel and seeds denotes 25 to 35% of the weight of processed apples [7]. The pomace produced from processed apple after juice extraction was 1.3 million tonnes and approximately 10000 tonnes is used [8] and left over AP thrown in the field causing harmful to environment [9]. AP is rich in carbohydrates (48-62%), proteins (4.45-5.67%), pectin and other nutrients [7]. Apple fruit processing industries are facing problems in the disposal of apple waste generated during pre and post-processing of apple fruits. Besides environmental pollution on dumping sites, it also poses possible health hazards due to growth of the undesirable microbes. Value addition of AP waste will not only reduce the environmental pollution, but also give the additional returns to apple processing industries. Although for many years, it is regarded as waste; at present, AP is being utilized for the value addition of different products [7-8].

In most of the countries, food grains are utilizing for mass multiplication of entomopathogenic fungi and other beneficial fungi including *Trichoderma harzianum*, *T. viridae* etc., for mass production. In in India, bio-pesticide production labs are using molasses from sugarcane for mass multiplication of beneficial fungi due to lack of agricultural waste. Development of cost-effective mass production technology is a prerequisite for beneficial fungi for formulation. Any fungi requires rich source of carbohydrates, nitrogen and neutral to alkaline pH for better growth and spore yield. AP is highly acidic, rich in carbohydrates but lacks nitrogen source for the growth of fungi. Therefore, it is necessary to supplement the AP with different nitrogen sources, water and modify the pH for better growth and spore production of *T. harzianum*. Therefore, present study planned with the primary aim of standardizing methodology for the multiplication of *T. harzianum* using AP as a substrate.

MATERIALS AND METHODS

Collection and preparation of AP powder

Apple fruit waste procured from Fruit Processing Plant, Parwanoo, H.P. and dried in shade under laboratory for 20 days and then powdered for experimental purpose.

Maintenance of *T. harzianum* culture

Pure culture of *T. harzianum* maintained in the Entomology laboratory on potato dextrose agar (PDA) as and when required for experimental work.

Effect of addition of different nitrogen sources in AP for spore production of *T. harzianum*

About 10g of AP powder was taken in 250 ml conical flasks, 0.5g of calcium carbonate was added in different concentrations (0.5, 1, 2, 3 and 4 g) of

ammonium sulphate [$(NH_4)_2 SO_4$], sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) separately in different flasks. All flasks were autoclaved at 121°C for 15 minutes and kept at room temperature (25-27 °C) for 24 hours to check the contamination, then inoculated with 1 ml spore suspension of *T. harzianum* under aseptic conditions. The culture flasks incubated at 27°C for ten days and then the spore yield (no. of spores/ml) was calculated after harvesting the mycelia. There were five treatments, and each treatment replicated five times.

Effect of addition of water in AP for spore production of *T. harzianum*

About 10g of AP powder was taken in 250 ml conical flasks; 0.5g of calcium carbonate was added in different concentrations of water (15 ml, 20 ml, 30 ml, and 40 ml) separately. The mixture mixed properly by using a glass rod and kept for one hour. All flasks were autoclaved at 121°C for 15 minutes and kept at room temperature (25-27°C) for 24 hours to check the contamination, then inoculated with 1 ml spore suspension of *T. harzianum* under aseptic conditions. The culture flasks incubated at 27°C for ten days and then the spore yield (no. of spores/ml) was calculated after harvesting the mycelia. There were four treatments, and each treatment replicated five times.

Effect of pH in AP for spore production of *T. harzianum*

About 10g of powdered AP taken in conical flask in which 40 ml distill water added to make slurry. 1N sodium hydroxide (NaOH) or hydrochloric acid (HCl) was added in the flasks to adjust the pH 6, 7, 8 and 9. All flasks autoclaved at 121°C for 15 minutes and kept at room temperature (25-27 °C) for 24 hours to check the contamination, then inoculated with 1 ml spore suspension of *T. harzianum* under aseptic conditions. The culture flasks incubated at 27°C for ten days and then the spore yield (no. of spores/ml) calculated after harvesting the mycelia. There were four treatments and each treatment replicated five times.

Harvesting of *T. harzianum* and calculation of spore production

T. harzianum multiplied AP medium for ten days in the flasks and then spores harvested as per the method adapted [10].

Statistical analysis

The data on spore yield analysed by one way analysis of variance (ANOVA) using SPSS statistical software, version 16 and means compared by Tukeys test.

RESULTS

Effect of supplementing nitrogen sources in AP for spore production of *T. harzianum*

Effect of addition of ammonium sulphate, sodium nitrate, potassium nitrate and ammonium nitrate in AP on spore production of *T. harzianum* presented in Table 1, Figure 1 and Figure 1a-d. All the nitrogen sources at different concentrations showed the growth significantly.

Ammonium sulphate: Among different concentrations of ammonium sulphate evaluated, addition of 0.50g of ammonium sulphate in AP showed significantly ($F_{4,24} = 45.55$; $p<0.0001$) higher spore production (10.82 ± 0.35 crorers/ml) after 10 days of inoculation and was at par with 1.0g (10.10 ± 1.05 crore spores/ml) followed by 2, 3 and 4 g/ml (4.32 ± 0.17, 3.19 ± 0.75 and 2.20 ± 0.09 crore spores/ml) repectively.

Sodium nitrate: Among different concentrations of sodium nitrate evaluated, addition of 1.0 g sodium nitrate in AP showed significantly ($F_{4,24} = 173.15$; $p<0.0001$) higher spore production (14.80 ± 0.42 crore spores/ml) after 10 days of

inoculation and was followed by 2, 3, 0.5 and 4 g/ml (12.69 ± 0.17, 10.57 ± 0.10, 9.63 ± 0.28 and 6.04 ± 0.14 crore spores/ml) repectively.

Potasium nitrate: Among different concentrations of potasium nitrate evaluated, addition of 3.0 g potasium nitrate in AP showed significantly ($F_{4,24}$ =184.88; p<0.0001) higher spore production (11.02 ± 0.18 crore spores/ml) after 10 days of inoculation and was at par with 1.0g (10.45 ± 0.16 crore spores/ml) followed followed by 0.5, 2.0 and 4 g/ml (9.15 ± 0.09, 7.13 ± 0.18 and 6.60 ± 0.07 crore spores/ml) repectively.

Ammonium nitrate: Among different concentrations of potasium nitrate evaluated, addition of 4.0 g potasium nitrate in AP showed significantly ($F_{4,24}$ =132.34; p<0.0001) higher spore production (13.17 ± 0.31 crore spores/ml) after 10 days of inoculation and was followed by 3.0, 2.0, 1.0 and 0.50 g/ml (11.34 ± 0.11, 8.80 ± 0.33, 7.97 ± 0.30 and 5.83 ± 0.08 crore spores/ml) repectively.

Effect of addition of water in AP for spore production of *T. harzianum*

Effect of addition of water in AP on spore production of *T. harzianum* is presented in Table 2,

Table 1
Effect of nitrogen sources on AP for spore production of *T. harzianum*

	No. of spores/ml (in crores)			
Concn (g)	$(NH_4)_2SO_4$	$NaNO_3$	KNO_3	NH_4NO_3
0.5	10.82 ± 0.35 a	9.63 ± 0.28 c	9.15 ± 0.09 b	5.83 ± 0.08 d
1.0	10.10 ± 1.05 a	14.80 ± 0.42 a	10.45 ± 0.16 a	7.97 ± 0.30 c
2.0	4.32 ± 0.17 b	12.69 ± 0.17 b	7.13 ± 0.18 c	8.80 ± 0.33 c
3.0	3.19 ± 0.75 b	10.57 ± 0.10 c	11.02 ± 0.18 a	11.34 ± 0.11 b
4.0	2.20 ± 0.09 b	6.04 ± 0.14 d	6.60 ± 0.07 c	13.17 ± 0.31 a
F value	$F_{4,24}$ = 45.55; p<0.0001	$F_{4,24}$ = 173.15; p<0.0001	$F_{4,24}$ = 184.88; p<0.0001	$F_{4,24}$ = 132.34; p<0.0001

"Means followed by the same alphabetical letters within column are not significantly different by Tukeys test"

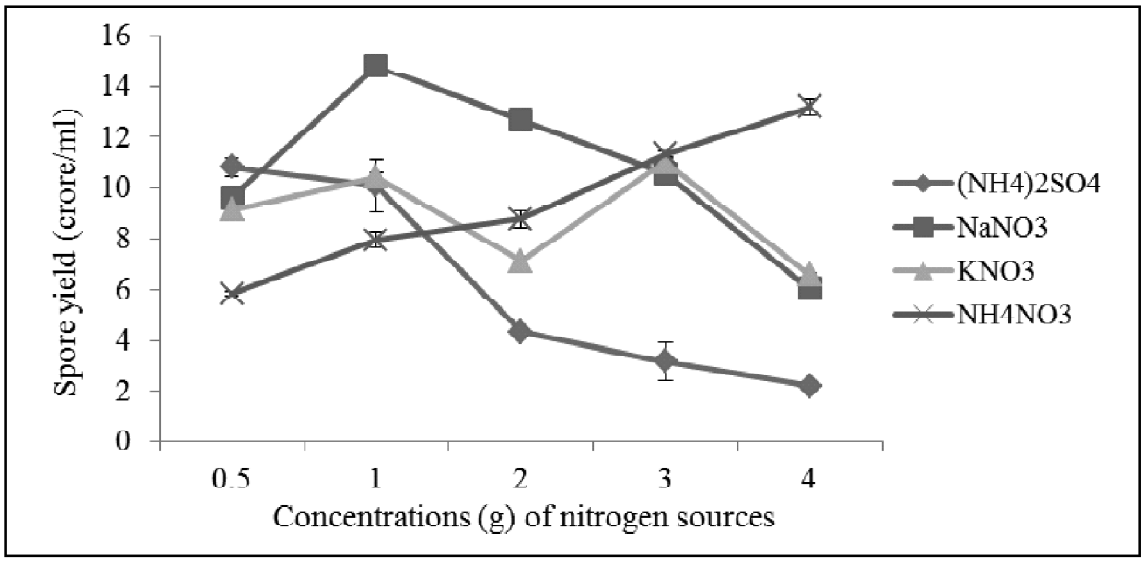

Figure 1: Effect of nitrogen sources on AP for spore yield of *T. harzianum*

Figure 1a

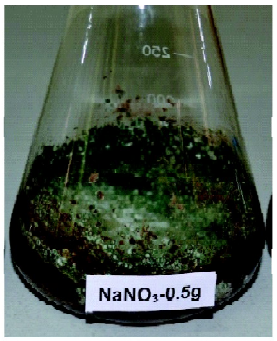

Figure 1b

Figure 1c

Figure 1d

Figure 1a-d: AP supplemented with NH_4NO_3 (a), $NaNO_3$ (b), KNO_3 (c) and $(NH4)_2SO_4$ (d) for the growth of *T. harzianum*

Figure 2 and Figure 2a. Results showed that, addition of 50 ml of water to AP showed significantly ($F_{3, 19}$ = 419.23; p<0.0001) higher spore production of *T. harzianum* (9.65 ± crore spores/ml) after 10 days of inoculation and followed by 40 and 30 ml (8.30 ± 0.22 and 4.57 ± 0.07 crore spores/ml) respectively. Addition of 20 ml of water to AP showed significantly less spore production (2.86 ± 0.05 crore spores/ml).

Effect of pH in AP for spore production of *T. harzianum*

Effect of different pH on spore yield of *T. harzianum* multiplied on AP medium presented in Table 3 and Figure 3. Among different pH evaluated, spore production of *T. harzianum* was significantly ($F_{3, 19}$ = 969.99; p<0.0001) higher in pH 9 (10.53 ± 0.08 crore spores/ml) and was followed by pH 8 (8.28 ± 0.05 crore spores/ml) and pH 7 (5.83 ± 0.12 crore spores/ml) as compared to acidic pH. The spore production was significantly less in acidic pH (4.66 ± 0.05 crore spores/ml).

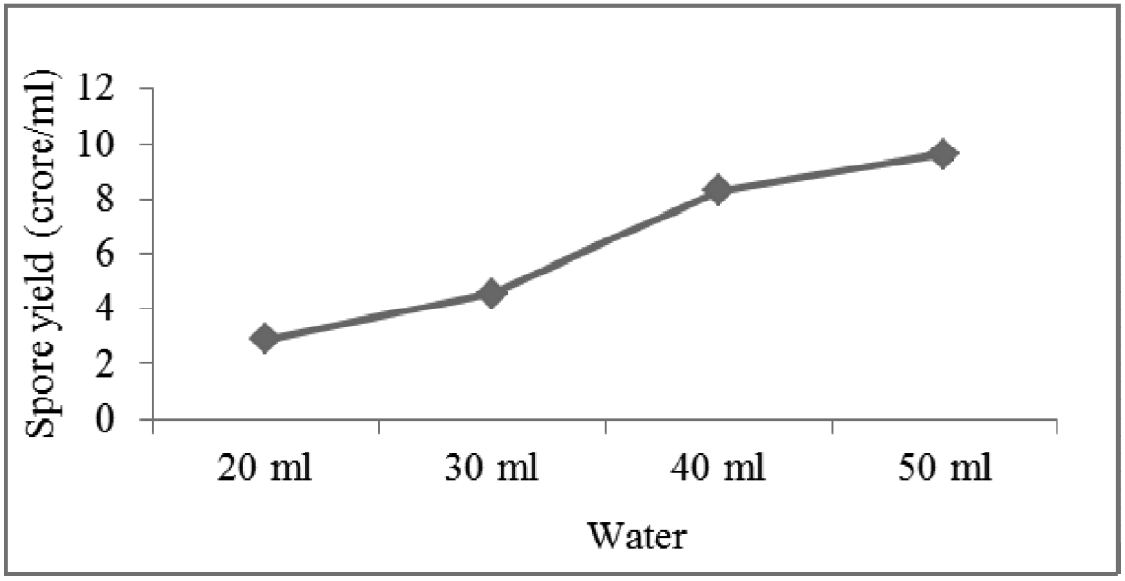

Figure 2: Effect of addition of water in AP for spore yield of *T. harzianum*

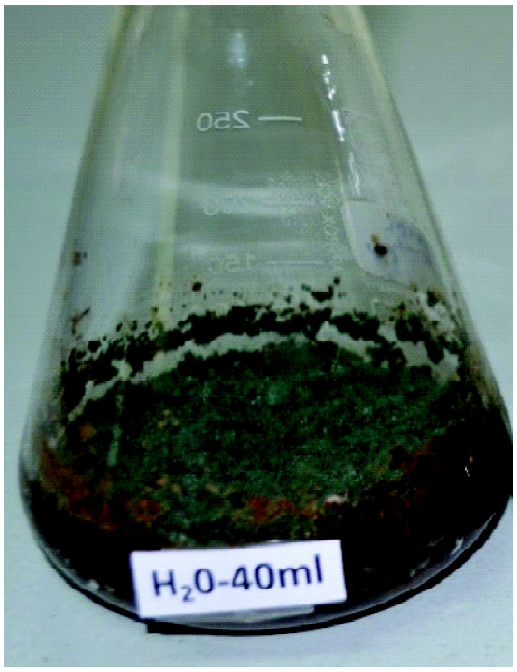

Figure 2a: Addition of water in AP for the growth of *T. harzianum*

Table 2
Effect of water on AP for spore production of *T. harzianum*

Concn.	*No. of spores/ml (in crores)*
20 ml	2.86 ± 0.05 d
30 ml	4.57 ± 0.07 c
40 ml	8.30 ± 0.22 b
50 ml	9.65 ± 0.20 a
F value	$F_{3,19}$ = 419.23; p<0.0001

"Means followed by the same alphabetical letters within column are not significantly different by Tukeys test"

Table 3
Effect of pH on AP for spore production of T. harzianum

pH	*No. of spores/ml (in crores)*
6	4.66 ± 0.05 d
7	5.83 ± 0.12 c
8	8.28 ± 0.05 b
9	10.53 ± 0.08 a
F value	$F_{3,19}$ = 969.99; p<0.0001

"Means followed by the same alphabetical letters within column are not significantly different by Tukeys test"

Effect of optimized concentrations of nitrogen sources, water and pH in AP for spore production of *T. harzianum*

The results of the optimized concentrations of nitrogen sources, water and pH for growth and spore production of *T. harzianum* in AP medium presented in Table 4 and Figure 4. Among them, addition of 0.5 g ammonium sulphate in AP powder showed significantly higher spore production of *T. harzianum* (10.85 ± 0.17 crore spores/ml). The other nitrogen sources viz., sodium nitrate (9.94 ± 0.22 crore spores/ml), potassium nitrate (9.45 ± 0.10 crore spores/ml) and water (9.57 ± 0.26 crore spores/ml) were at par with each other followed by ammonium nitrate (7.97 ± 0.11 crore spores/ml) and pH 10 (7.42 ± 0.08 crore spores/ml).

Table 4
Effect of optimized nitrogen sources, water and pH on AP for spore production of T. harzianum

Treatment s	*Concn.*	*No. of spores/ml (in crores)*
KNO_3	0.50 g	9.45 ± 0.10 b
NH_4NO_3	1.0 g	7.97 ± 0.11 c
$NaNO_3$	0.50 g	9.94 ± 0.22 b
$(NH_4)_2SO_4$	0.50 g	10.85 ± 0.17 a
Water	40 ml	9.57 ± 0.26 b
pH	10	7.42 ± 0.08 c
	F value	$F_{5,29}$ = 56.83; p<0.0001

"Means followed by the same alphabetical letters within column are not significantly different by Tukeys test"

DISCUSSION

Carbon and nitrogen play a major role in the growth and multiplication of any fungi by using solid as substrate. Carbon is already available in the apple pomace waste but less nitrogen is one of the constraint for the growth of fungi. In the present study, addition of water, supplementing different nitrogen sources viz., ammonium sulphate, sodium nitrate, potassium nitrate and ammonium nitrate in AP, adjusting the different pH (neutral to alkaline) and effect of addition of water which directly

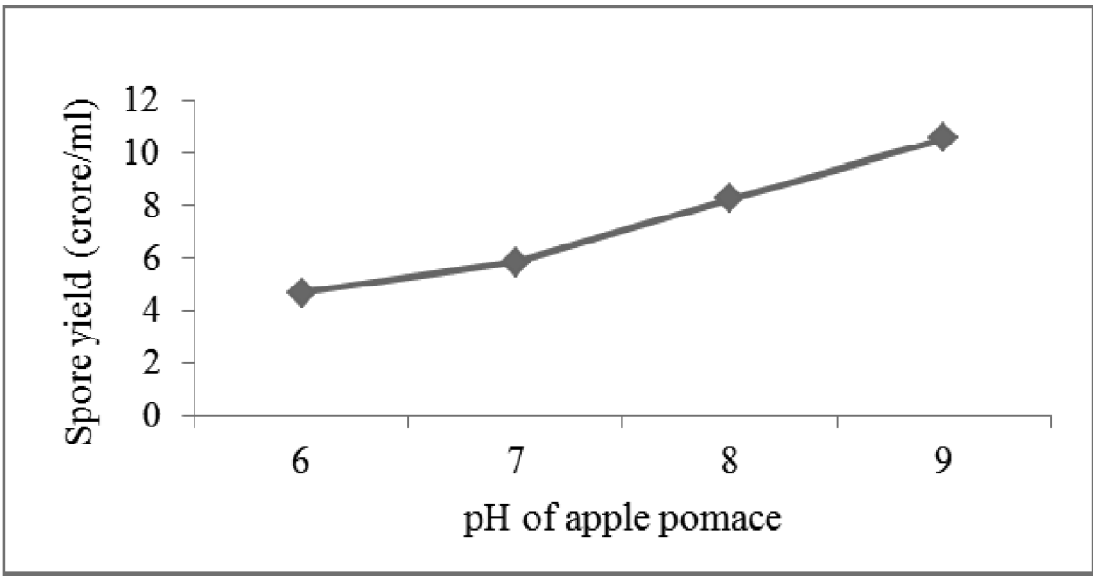

Figure 3: Effect of pH in AP for spore yield of *T. harzianum*

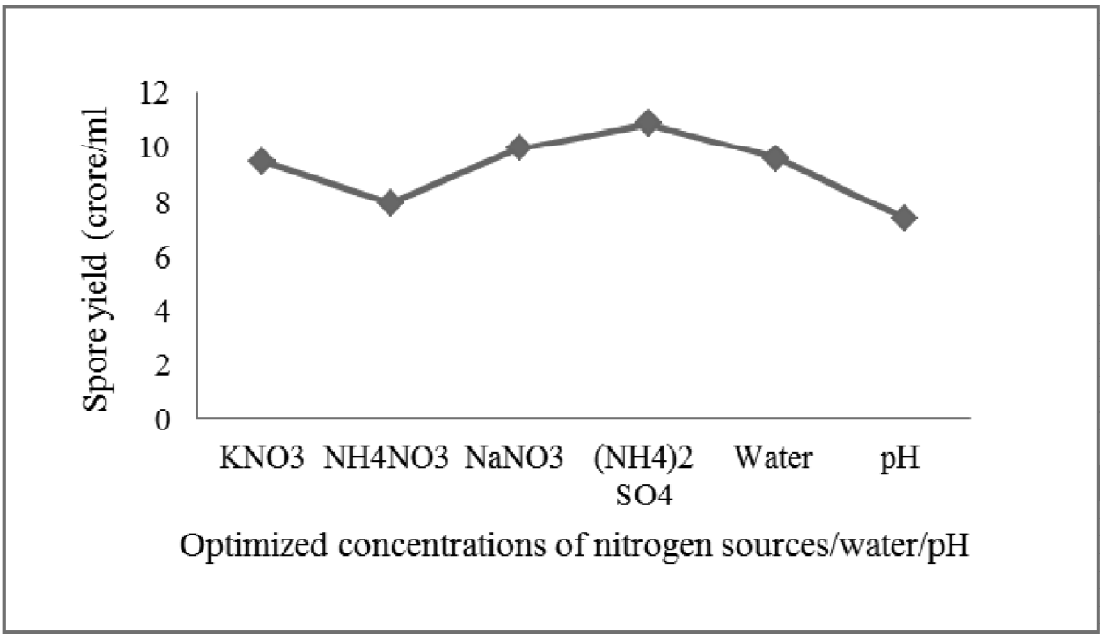

Figure 4: Effect of optimized sources of nitrogen, water and pH for spore yield of *T. harzianum*

influences the growth and spore production of *T. harzianum* after 10 days of inoculation. *T. harzianum* and other beneficial fungi prefer to grow better in alkaline pH as compared to neutral. AP has acidic pH (3 to 3.7) due to organic acid content and therefore it is necessary to add calcium carbonate to neutralize AP medium. Due to acidic nature, the AP adjusted with pH by adding HCl and NaOH and then reported better growth and spore production in alkaline pH as compared to acidic and neutral.Yeast mold culture media adjusted with pH 5 to 9 and water activity (>0.99) showed better germination, sporulation and growth [11]. Growth rate and biomass of *B. bassiana*-G07 multiplied on

yeast extract peptone glucose agar was higher in neutral to alkaline pH [12].

The moisture present in any media/substrate play significant role for the fungal growth [13-14]. Higher moisture content in AP medium increases the aerial mycelia. *T. harzianum* was not shown any growth in AP in the present study if water was not added due to lack of moisture and showed maximum growth and spore yield when 4-5 ml of water added per gram of AP. In present study, addition of water (5 ml/g of AP) showed better growth of *T. harzianum*. Similarly, the supplementing the different nitrogen sources of ammonium sulphate, sodium nitrate, potassium nitrate and ammonium nitrate at different concentrations (0.05-0.4 g/g of AP) showed better growth and spore yield of *T. harzianum*. Present results are in agreement with the findings of Zheng and Shetty [14], who reported the addition of 0.05 g of calcium carbonate, 2 to 5 ml of water and 0.05g of ammonium nitrate per gram of AP showed optimum fungal growth of *Trichoderma* spp, *Penicillium* and *Rhizopus* species. In another study Zheng and Shetty [15] reported promising growth of *T. harzianum*, *T. pseudokoningii*, Penicillium isolates, and Rhizopus in cranberry pomace supplemented with calcium carbonate, water, and ammonium nitrate or fish protein hydrolysate/gram of pomace.

Addition of carbon and nitrogen in basic AP medium showed maximum biomass yield and carotenoid production by *Micrococcus* sp. Addition of sodium nitrate to apple pomace media showed the highest production of biomass of *Micrococcus* sp. [16]. The combination of AP and powder of cotton seed with ammonium sulphate and potassium phosphate reported as optimum medium for the growth of strains of *Aspergillus niger* [17]. *T. harzianum* showed maximum spore count on bio-waste of carrot (3.14×10^7) as compared to mango chukandar banana and papaya (3.07, 2.97, 2.94 and 2.86×10^7 spores/ml, respectively) [18]. In a similar study, vegetable waste reported maximum spore production of *Metarhizium anisopliae* (8.8×10^7 spores/g) and *T. longibrachiatum* (7.96×10^7 spores/g) after 15 days of incubation as compared to rice straw, sugarcane bagasse, coconut coir and corn cob [19]. The media contains apple pomace, strawberry pomace, rind of onion and rapeseed meal reported better growth *Trichoderma* sp [20].

CONCLUSION

Addition of water, modifying the pH, supplementing different nitrogen sources viz., ammonium nitrate, reported more spore production of *T. harzianum*. Therefore, the AP can be use as a solid substrate for mass production of beneficial fungi which can be used for soil application to control soil borne pathogens.

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REFERENCES

[1] Alter, J.A., and Vandenberg, J.J.D., (2000). Factors that influencing the infectivity of isolates of *Paecilomyces fumosoroseus* against diamond back moth. *Journal of invertebrate pathology*, 78, 31–36.

[2] Gindin, G., *et al.*, (2000). Pathogenicity of *Verticillium lecanii* to different developmental stages of the silver leaf whitefly, *Bemisia argentifolii*. *Phytoparasitica*, 28, 229–239.

[3] Wang, L., *et al*., (2004). Time dose mortality modelling and virulence indices for six strains of *Verticillium lecanii* against sweet potato whitefly, *Bemisia tabaci* (Gennadius). *Journal of applied entomology*, 128, 494–500.

[4] Goettel, M.S., *et al*., (2008). Potential of *Lecanicillium* spp. for management of insects, nematodes and plant diseases. *Journal of invertebrate pathology*, 98, 256–61.

[5] Bharathi, H.T., (2005). Isolation and characterization of entomopathogenic fungi and their effectiveness. Ph.D. thesis submitted to the University of Agricultural Sciences, Dharwad (India) p. 129.

[6] Anonymous (2017). Hoticultultural Statistics at a Glance 2017, Horticultural Statistics Division, Department of Agriculture, Cooperation and Farmers Welfare, Ministry of Agriculture and Farmers Welfare, Government of India.

[7] Joshi, V.K. and Attri, D., (2006). Solid state fermentation of apple pomace for the production of value added products. *Natural product radiance*, 5, 289–296.

[8] Shalini, R., and Gupta, D.K., (2010). Utilization of pomace from apple processing industries: a review. *Journal of food science and technology*, 47(4), 365–371.

[9] Kaushal, N.K., Joshi, V.K., Sharma, R.C., (2002). Effect of stage of apple pomace collection and the treatment on the physical-chemical and sensory qualities of pomace papad (fruit cloth). *Journal of food science and technology*, 39, 388–393.

[10] Reddy, S.G.E. and Sahotra, S., (2018). Multiplication entomopathogenic fungus (*Lecanicillium lecanii*) on apple pomace and its toxicity against aphid (*Aphis craccivora*). *Toxin reviews*. DOI:10.1080/15569543.2018.1504222.

[11] Aguirre, N., *et al*., (2009). Effect of pH and water activity over *Nomuraea rileyi* (Hyphomycetes) development. *Revista colombiana de entomología*, 35 (2),138–144.

[12] Otgonjargal, K., *et al*., (2015). Optimum and tolerance pH range, optimal temperature of the local strain *Beauveria bassiana* G-07. *International Journal of Agriculture Innovations and research*, 4 (3), 578–580.

[13] Cannel, E. and Moo-Young, M., (1980). Solid state fermentation systems. *Process biochemistry*, 15 (5), 2–7.

[14] Zheng, Z.X., and Shetty, K., (1998a). Cranberry processing waste for solid state fungal inoculant production. *Process biochemistry*, 33 (3), 323–329.

[15] Zheng, Z.X., and Shetty, K., (1998b). Solid-state production of beneficial fungi on apple processing wastes using glucosamine as the indicator of growth. *Journal of agricultural and food chemistry*, 46 (2),783–787.

[16] Attri, D. and Joshi, V.K., (2005). Optimization of apple pomace based medium and fermentation conditions for pigment production by Micrococcus species. *Journal of Scientific and Industrial Research*, 64(8), 598–601.

[17] Sun, Z.T., *et al*., (2009). Bioconversion of apple pomace into a multienzyme bio-feed by two mixed strains of *Aspergillus niger* in solid state fermentation. *Electronic journal of biotechnology,* 12(1). DOI: 10.4067/S0717-34582009000100002.

[18] Ghosh, S.K. and Pal, S., (2015). Mass multiplication of entomopathogenic fungi using agricultural waste. *Newyork science journal*, 8, 82–86.

[19] Ganaie, M.A. and Khan, T.A., (2013). *In vitro* studies of biowastes on growth and sporulation of fungal bioagents. *African journal of agricultural research,* 8 (37), 4660–4663.

[20] Smolinska, U., *et al*., (2014). The use of agro-industrial wastes as carriers of *Trichoderma* fungi in the parsley cultivation. *Scientia horticulturae* 179, 1–8.