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Research Article

Qualitative and Quantitative Analysis of Phylloplane Mycoflora of *Stevia Rebaudiana* Bertoni

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Abstract: The aim of the present investigation is to study the fungal flora of young and mature leaves of *S. rebaudiana*. Young and mature leaves of *S. rebaudiana* were used for isolation of fungi. Phylloplane mycoflora were isolated from leaves of *S. rebaudiana* by using three methods i.e. dilution plate method, leaf impression method and leaf washing method. Dilution plate method and leaf washing method were applied for quantitative analysis and leaf impression method for qualitative analysis. A total of 35 fungal species comprising Zygomycetes, Ascomycetes and Deuteromycetes including sterile forms were isolated during the course of investigation. A total of thirteen fungal species viz. *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Penicillium cyclopium*, *P. chrysogenum*, *Cladosporium elatum*, *C. cladosporioides*, *Mucor etc.* were isolated from mature leaves. Eleven fungal species were found by leaf impression method, eight by leaf washing method and six by dilution plate method. *Aspergillus*, *Penicillium* and *Cladosporium* species were found to be dominant among all the species. Phylloplane fungal population on mature leaves of *S. rebaudiana* was 1.68×10^2 /cm^2 leaf area. Thus, the outcome of study presents a clear picture about fungal population and diversity in phylloplane of *S. rebaudiana* mature leaves.

INTRODUCTION

Stevia rebaudiana Bertoni, belonging to the family Compositae, is a sweet perennial shrub native to South America. The main sweet component of the leaves of *S. rebaudiana* Bertoni is stevioside [1]. The phylloplane, the surface of plant leaves is a complex terrestrial habitat that is characterized by a variety of microorganisms including bacteria, filamentous fungi and yeast. Phylloplane fungi have been poorly studied as compared to endophytes, saprobes and pathogenic fungi. The interest shown in the last few years in the study of phyllplane microbes is due to their interactions with plants, herbivores and pathogens living on leaves which may be involved in the plant immunity system, reabsorption of organic and mineral matters from leachates, redistribution of nutrients prior to leaf fall and participation in the primary degradation of plant tissues [2-5].

Addition of yeasts and fungi evident that the overall phyllosphere microbiota is sufficiently large to influence global carbon and nitrogen cycles [6-7]. Leaf surface harbours certain human pathogenic microorganisms, which proved to be hazardous for public health. The presence of phylloplane fungi on the leaves of pasture plant influences the health of grazing mammals. *Pithomyces chartarum,* the causal organism of facial eczema of sheep, *Myrothecium verrucaria* and *Phoma glumarum* were found to be toxic to cattle [8-9]. *Stachybotrys chartarum* competes and suppresses other microorganisms through biosynthesis and production of trichothecenes, e.g. satratoxines, verrucarins, trichoverrins and atranones, but some of them have proven to have toxicological impact on the environment and human and animal health [10-11]. Phylloplane mycoflora varies in size and diversity depending on the influence of numerous biotic and abiotic factors which affect their growth and survival [12]. Phylloplane microorganisms compete for nutrients, space or antagonize each other by production of antibiotics, by mycoparasitism, lysis of other microorganisms or they may stimulate the leaf to produce phytoalexins [13].

Surface microflora interact with leaf pathogens and also impact disease development [14]. The influence of surface microflora has been further enhanced by recent studies showing the existence of cyclic pattern of appearance of air phylloplane litter soil microflora [15]. In the present investigation the studies were undertaken to gather the information of phylloplane mycoflora of *S. rebaudiana*.

MATERIALS AND METHODS

Sample collection

Young and mature leaves of *S. rebaudiana* were removed at random from plants at Dr. Shushila Tiwari herbal garden,

Muni-ki-reti, Rishikesh, Uttarakhand and placed separately in different sterilized plastic boxes.

Isolation of phylloplane fungi

In the present investigation three cultural techniques were tried i.e. dilution plate method [16], leaf washing method [17] and leaf impression method [18] to obtain clear picture of phylloplane mycoflora and provide statistically significant results.

Record of fungal flora

The observations of incubated petriplates were taken after seven days. A complete record of data of number of colonies of each species was kept. The percentage of abundance and percentage of frequency of each species occurring on different plates was calculated as follows:

$$\text{Percentage of abundance} = \frac{\text{No. of colonies of individual species in all plates studied}}{\text{Total number of colonies of all the species}} \times 100$$

$$\text{Percentage of frequency} = \frac{\text{No. of observations in which a species appeared}}{\text{Total number of observations}} \times 100$$

$$\text{Propagules/cm}^2 = \frac{\text{Total number of spores in one ml}}{\text{Total area of 40 discs} \times 2} \times 100$$

Identification of fungi

The isolated fungi were maintained in pure culture and identification of fungi was done by lactophenol cotton blue staining and further observing them under binocular microscope at 10X, 40X and 100X magnifications. Fungi were identified on the basis of their morphology and culture characterstics. The observations were compared with those of the standard characteristics given by Gilman, Ellis, Subramanian, Barnett and Hunter etc. [19-25]. These fungal species were further confirmed from Forest Pathology Division, Forest Research Institute, Dehradun.

Statistical analysis

The data were statistically analysed by using Microsoft Office Excel 2007.

RESULTS AND DISCUSSION

The phylloplane mycoflora of *S. rebaudiana* was analysed quantitatively as well as qualitatively in 2013 crop season by employing three cultural techniques. Dilution plate method and leaf washing method was used for quantitative estimation of mycoflora and leaf impression method for qualitative estimation of mycoflora. All three methods were found helpful in presenting the spectrum of different fungi on the phylloplane (Fig 1).

The quantitative analysis of phylloplane mycoflora is represented in table 1 and 3. Dilution plate studies (Table 1) revealed that the number of fungal propagules/cm^2 on young leaves was less than mature leaves. In the first sampling, leaves harbour rich mycoflora which decreased in second sampling while third sampling gave the highest counts.

Qualitative studies on phylloplane mycoflora of *S. rebaudiana* showed the presence of 20 and 24 fungal forms belonging to 15 and 16 genera in all three samplings from young and mature leaves respectively. A total of 28 fungal forms were isolated from phylloplane of *S. rebaudiana* by leaf impression method (Table 2). Through the leaf impression method *Aspergillus terreus*, *Penicillium notatum, P. oxalicum*, *Scytalidium* sp., *Stachybotrys chartarum*, and black sterile mycelia were found only on mature leaves while *Emericella nidulans* restricted to young leaves. *Alternaria tenuissima, Aspergillus flavus, Cladosporium cladosporioides* and *Periconiella* sp. had 100% frequency on young leaves while *Aspergillus fumigatus, A. niger* and *C. cladosporioides* on mature leaves.

Table 1: Variation in density (propagules/cm^2) of phylloplane mycoflora of *S. rebaudiana* obtained by dilution plate method

Type of leaf	Sampling no./ sampling date/age (days)		
	I	II	III
	06/03/13	20/06/13	30/09/13
	56	162	264
Young	127.94	97.79	129.4
Mature	153.68	123.60	172.06

ANOVA single factor

Source of variation	Sum of squares	Degree of freedom	Mean of squares	F-value	P-value	F crit
Between Groups	1738.75	2	869.37	1.656697	0.327558	9.552094
Within Groups	1574.29	3	524.76			
Total	3313.04	5				

Table 2: Composition of phylloplane mycoflora of young and mature leaves of *S. rebaudiana* obtained by leaf impression method

S.N.	Name of microorganism	Sampling no., sampling date and age (days)						Frequency (%)	
		I 06/03/13 56		II 20/06/13 162		III 30/09/13 264			
		Y	M	Y	M	Y	M	Y	M
1.	*Alternaria tenuissima*	+	+	+	+	+	-	100	66.67
2.	*Aspergillus flavus*	+	+	+	-	+	+	100	66.67
3.	*A. fumigatus*	-	+	-	+	-	+	-	100
4.	*A. niger*	+	+	-	+	+	+	66.67	100
5.	*A. terreus*	-	-	-	+	-	-	-	33.33
6.	*Botrytis cinerea*	-	-	-	-	+	+	33.33	33.33
7.	*Chaetomium globosum*	-	-	-	-	+	+	33.33	33.33
8.	*Cladosporium cladosporioides*	+	+	+	+	+	+	100	100
9.	*Curvularia lunata*	-	-	-	-	+	+	33.33	33.33
10.	*Emericella nidulans*	-	-	+	-	-	-	33.33	-
11.	*Histoplasma* sp.	-	-	-	-	-	+	-	33.33
12.	*Microsporum* sp.	-	-	-	-	+	+	33.33	33.33
13.	*Mucor hiemalis*	+	+	+	-	-	-	66.67	33.33
14.	*Paecilomyces variotii*	-	-	-	-	+	+	33.33	33.33
15.	*Penicillium cyclopium*	+	+	+	+	-	-	66.67	66.67
16.	*P. expansum*	+	+	-	-	-	-	33.33	33.33
17.	*P. notatum*	-	-	-	-	-	+	-	33.33
18.	*P. oxalicum*	-	-	-	-	-	+	-	33.33
19.	*P. purpurogenum*	+	+	+	-	-	-	66.67	33.33
20.	*Penicillium* sp.	-	-	-	-	+	-	33.33	-
21.	*Periconiella* sp.	+	+	+	+	+	-	100	66.67
22.	*Rhizopus oryzae*	-	-	-	+	+	+	33.33	66.67
23.	*Scytalidium* sp.	-	-	-	+	-	-	-	33.33
24.	*Sporobolomyces roseus*	-	-	+	+	+	+	66.67	66.67
25.	*Stachybotrys chartarum*	-	+	-	-	-	-	-	33.33
26.	*Syncephalastrum racemosum*	-	-	-	+	+	+	33.33	66.67
27.	Sterile Black mycelia	-	-	-	-	-	+	-	33.33
28	Sterile White mycelia	+	+	+	-	-	+	66.67	66.67
	Total	10	12	10	11	14	17		

(Y= Young, M= Mature, + = present, - = absent)

A variety of methods used for the isolation of phylloplane mycoflora of *S. rebaudiana* showed that leaf impression method yielded maximum species followed by leaf washing method and dilution plate method. Dilution plate method revealed a total of 19 fungal species from 12 genera in which 13 fungal species belonging to 8 genera on young leaves and 15 species belonging to 9 genera on mature leaves (Table 3). Leaf washing studies revealed a total of 21 fungal forms belonging to 12 species isolated from leaves of *S. rebaudiana* leaves in which 16 species belonging to 8 genera on young leaves and 14 species belonging to 9 genera on mature leaves.

Qualitatively the isolated mycoflora was the same except *Aspergillus ochraceus*, *A. terreus, Trichoderma hamatum, E. nidulans*, *Histoplasma* sp., *Sporothrix* sp. *S. chartarum*, *Trichophyton* sp. and Sterile Black mycelia. Mature leaves harbour *A. ochraceus, A. terreus*, *T. hamatum*, *Histoplasma* sp., *Sporothrix* sp., *S. chartarum*, *Trichophyton* sp. and Sterile Black mycelia while *E. nidulans* and *Penicillium* sp. were present only on young leaves.

Table 3: Distribution of phylloplane mycoflora on *S. rebaudiana* obtained by three methods i.e. leaf impression, dilution plate and leaf washing method

S.N	Name of microorganism	Sampling no., sampling date and age of plant (days)																	
		I 06/03/13 56						II 20/06/13 162						III 30/09/13 264					
		L.I.		D.P.		L.W.		L.I.		D.P.		L.W.		L.I.		D.P.		L.W.	
		Y	M	Y	M	Y	M	Y	M	Y	M	Y	M	Y	M	Y	M	Y	M
1.	*Alternaria tenuissima*	+	+	-	-	-	-	+	+	-	-	-	-	+	-	10.85	5.75	-	-
2.	*Aspergillus awamori*	-	-	-	-	0.10	0.07	-	-	-	-	0.07	-	-	-	-	-	-	-
3.	*A. flavus*	+	+	10.12	-	0.07	0.15	+	-	-	-	0.25	0.3	+	+	15.6	12.08	0.15	-
4.	*A. fumigatus*	-	+	-	25.91	-	-	-	+	-	25.47	1.15	0.6	-	+	-	-	-	0.05
5.	*A. niger*	+	+	-	-	-	0.17	-	+	-	-	-	-	+	+	-	-	-	0.07
6.	*A. ochraceus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19.71	-	-
7.	*Aspergillus terreus*	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
8.	*Aspergillus* sp.	-	-	-	-	-	-	-	-	-	5.7	0.05	-	-	-	-	-	-	-
9.	*Botrytis cinerea*	-	-	-	-	-	-	-	+	-	-	0.05	-	+	+	-	-	0.15	-
10.	*Chaetomium globosum*	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
11.	*Cladosporium cladosporioides*	+	+	9.78	25.80	0.17	0.07	+	+	9.5	-	-	-	+	+	12.54	-	0.20	0.05
12.	*Curvularia lunata*	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	0.20	0.05
13.	*Emericella nidulans*	-	-	-	-	-	-	+	-	14.75	-	-	-	-	-	-	-	-	-
14.	*Histoplasma* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	0.02
15.	*Microsporum* sp.	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
16.	*Mucor hiemalis*	+	+	-	5	-	-	+	-	-	5.58	-	-	-	-	21.3	23.39	-	-
17.	*Paecilomyces variotii*	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
18.	*Penicillium cyclopium*	+	+	23.39	9.36	0.07	0.17	+	+	24.65	24.90	0.8	1.1	-	-	-	-	-	-
19.	*P. expansum*	+	+	30.26	-	0.13	-	-	-	-	-	-	-	-	-	-	-	-	-
20.	*P. notatum*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	12.5	2.08	0.05	0.2
21.	*P. oxalicum*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	0.05	-
22.	*P. purpurogenum*	+	+	14.67	17.55	0.1	-	+	-	25.54	14.2	0.25	0.45	-	-	-	-	-	-
23.	*Penicillium sp.*	-	-	-	-	-	-	-	-	-	-			+	-	-	-	-	-
24.	*Periconiella* sp.	+	+	9.78	5.85	0.13	0.13	+	+	12.25	-	0.25	0.25	+	-	-	-	-	-
25.	*Rhizopus oryzae*	-	-	-	-	-	-	-	+	-	-			+	+	-	-	-	-
26.	*Scytalidium lignicola*	-	-	2.05	3.51	-	-	-	-	-	-	0.03	-	-	-	-	8.85	-	-
27.	*Scytalidium* sp.	-	-	-	-	-	-	-	+	14.75	10.49	0.05	-	-	-	-	-	0.20	-
28.	*Sporobolomyces roseus*	-	-	-	-	-	-	+	+	-	13.32	0.25	0.65	+	+	20	16.4	0.15	0.02
29.	*Sporothrix* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05
30.	*Stachybotrys chartarum*	-	+	-	-	-	0.13	-	-	-	-	-	-	-	-	-	5.7	-	-
31.	*Syncephalastrum racemosum*	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-
32.	*Trichoderma hamatum*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.75	-	-
33.	*Trichophyton* sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09
34.	Sterile Black mycelia	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
35.	Sterile White mycelia	+	+	-	7.02	-	-	+	-	-	-	-	-	-	+	7.5	-	-	-
	Total	**10**	**12**	**7**	**8**	**7**	**8**	**10**	**11**	**6**	**7**	**11**	**6**	**14**	**17**	**7**	**9**	**8**	**9**

L.I= Leaf impression method; + = present; - = absent; D.P.= Dilution plate method (figures indicates percentage of abundance); L.W.= Leaf Washing method (figures indicates density/gm at 10^{-3} dilution); Y= Young; M= Mature

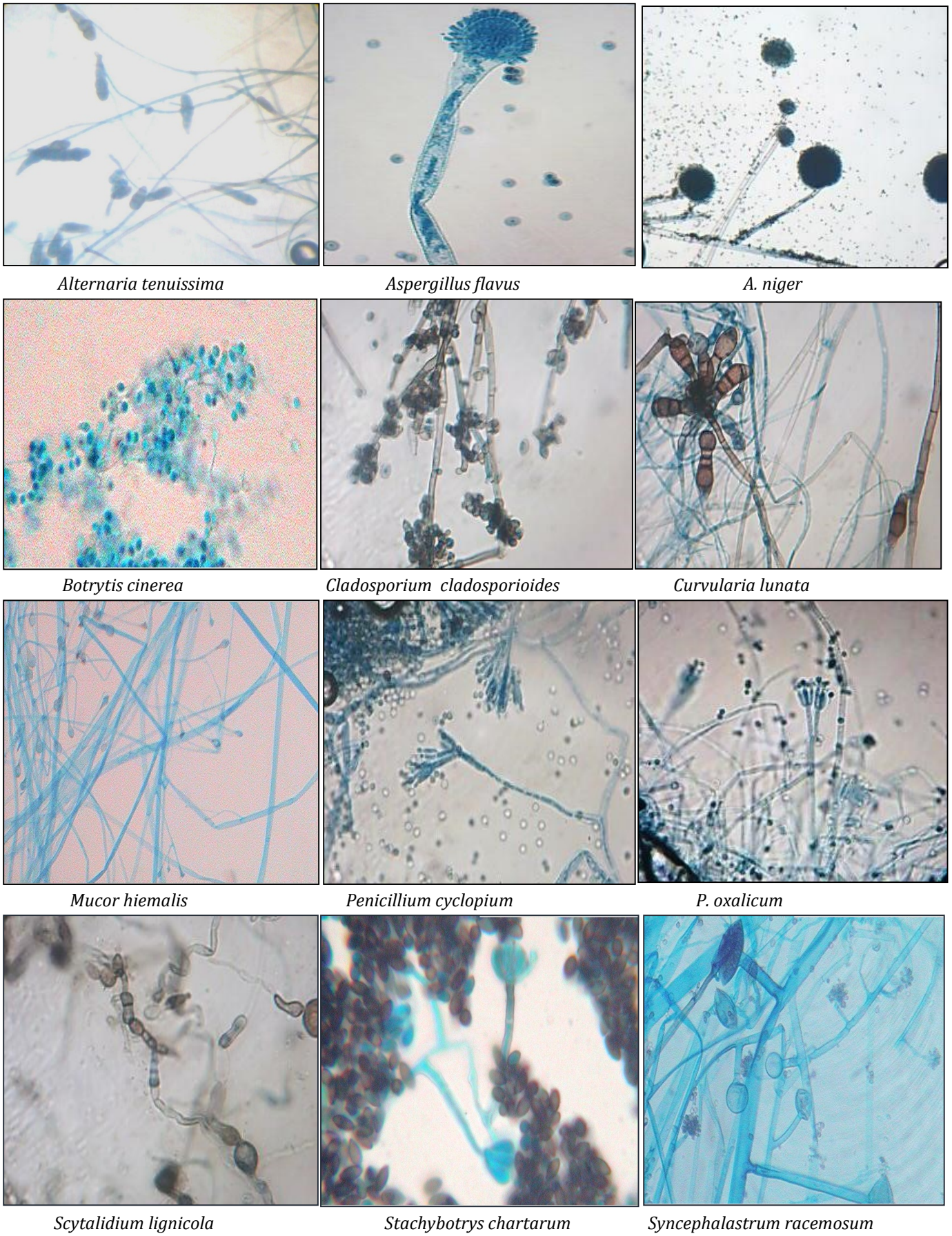

Fig. 1: Photographs of phylloplane fungi taken by binocular microscope with the help of Micro Image Projection System (MIPS) at 40X

It was observed that *A. terreus*, *Chaetomium globosum*, *Paecilomyces variotii*, *Penicillium oxalicum, Penicillium* sp., *Rhizopus oryzae* and *Syncephalastrum racemosum* have been isolated by leaf impression method, *A. ochraceus* and *T. hamatum* by dilution plate method and *Sporothrix* sp. and *Trichophyton* sp. by leaf washing method only. In this way a combination of all these techniques gave sufficient data regarding number, nature and occurrence of fungi on leaves.

In the present study, members of deuteromycetous forms were distributed on leaf surface in much larger proportions than other groups and constituted a major portion of the total fungal species isolated from beginning upto the end of growing season. Prevalence of these forms was in all probability due to their fast growing and high sporulating abilities and also their capacity to utilize a wide range of substances present on leaf surface. Consistent presence of *A. tennuisima*, *A. flavus* and *C. cladosporioides* is more or less in accordance with observation of Sahu et al [26]. Their occurrence in each season and at all stages of plants growth suggests that they are true colonizers of phylloplane of *Stevia* plants.

In present study, *Mucor hiemalis* though in insignificant proportion, was found to be associated with the phylloplane throughout while *R. oryzae* and *S. racemosum* appeared in second and third sampling. The occurrence of these forms may be directly related to the presence of their propagules in the air. These can utilize simple sugars formed as by product on leaf surface during metabolic activity of their phylloplane inhabitants and grow actively no sooner their spores settle on leaf surface [27].

The sterile or non-sporing mycelia were consistently recorded on leaf surface in the present study. Sterile White form was more prevalent on leaf surface but at the same time black sterile forms were also found to colonize the leaf surface. Consistent presence of sterile forms was also reported by Sahu et al [26].

CONCLUSION

The present investigation indicates that phylloplane mycoflora of Stevia is rich in quantity and quality. A combination of three methods of fungal isolation proved better than use of a single method. Maximum species of fungi was isolated by leaf impression method. In entire study the members of deuteromycetous constituted a major portion of the total fungal species.

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