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RESEARCH ARTICLE

QUALITATIVE ANALYSIS OF 2-ACETYL-1-PYRROLINE FROM THE RHIZOSPHERE FUNGAL SPECIES OF BASMATI RICE VARIETIES BY GC-FID

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ABSTRACT

Rhizosphere fungi of 12 basmati rice varieties collected from 11 different localities of North India were isolated, Total 207 fungal strains were obtained that were identified and their pure cultures are maintained. These strains were screened for 2AP synthesis; the principle basmati aroma compound on the synthetic medium proposed by Rungsardthong. 12 fungal species namely *Aspergillus terreus, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Nigrospora sphaerica, Nigrospora oryzae, Trichoderma* sps. *Penicillium* sps, *Rhizopus* oryzae, *Fusarium oxyporum, Monilia sitophila*, *Thielavia terricola* were found to synthesize 2AP. 2AP synthesizing fungus (*Aspergillus terreus)* was taken for qualitative analysis by GC-FID.

INTRODUCTION

(Hiltner, 1904) defined 'rhizosphere' as a zone of soil in which the microflora is influenced by the plant roots. Soil is loaded with a variety of microorganisms and plants depend for their nutrition and growth on soil microorganisms by means of their intimate relationship with the root system (Tagade, 2008). Soil microorganisms are capable of exerting beneficial effect either in culture and in protected environment and has a potential use in both agriculture and horticulture crops, which result in enhanced yield (Cook and Baker, 1983). There are many advantages in using microorganisms for flavour production such that the process is not subject to seasonal variation and climatic factors, product yields can be maximized by strain improvement or process optimization, production costs can be minimized by using cheap starting materials and the products obtained are considered as 'natural'.

Rhizosphere of scented rice varieties have also been screened to find out the fungal strain synthesizing the principle basmati aroma compound 2 acetyl-1-pyrroline (2AP). (Rungsardthong, 1995) reported *Aspergillus awamori* and *Acremonium nigricans* synthesizing 2AP. Moreover, (Nagsuk *et al*. 2003) isolated and identified 2AP in the cultures of *Aspergillus oryzae* TISTR 3088 and *Aspergillus oryzae* TISTR 3232. The Indian subcontinent has the 'natural gift' of basmati rice that has been accepted as the best scented, long and slender grain rice in the world markets and fetches high prices. North India is the rich sources of Basmati rice varieties. However, till date there are no reports of fungal isolates synthesizing 2AP from rhizosphere of Basmati rice varieties from India. The present work reports the fungal rhizosphere of Basmati rice varieties and the specific fungal strains synthesizing 2AP.

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MATERIALS AND METHODS

1. Collection of soil samples from basmati fields of North India

Rhizosphere soil samples from 13 basmati rice varieties cultivated in 11 different localities of North India were collected using the method of Dongmo and Oyeyiola, (2006). Each rhizospher soil sample was collected by carefully uprooting a plant and shaking the soil adhering to the roots into a sterile polythene bag and brought to the laboratory for further processing. The details of photosphere fungal isolates are given in Table. 1

Table 1. Details of the localities of collection and Basmati rice (*O. sativa* L.) varieties collected

Variety	Locality
Sharbati Basmati	Moradabad
Sharbati Basmati	Rampur
Sharbati Basmati	Pilibhit
Sharbati Basmati	Nainital
Golden Basmati	Nainital
Basmati 11 21 A	Udhamsingh Nagar
Basmati 11 21 B	Udhamsingh Nagar
Basmati Sugandha	Bijnor
Pusa Basmati	Muzaffarnagar
Double Chabi	Muzaffarnagar
Toda Basmati	Muzaffarnagar
Royal Bhog	Haridwar
Dehradun Basmati	Dehradun
Basmati 999	Saharanpur
Basmati CH30	Karnal
Karnal Local	Karnal

2. Culturing and isolation of rhizosphere mycoflora by serial dilution plate technique (Johnson and Curl, 1972)

From each basmati rice variety, the rhizosphere soil was used for isolation of mycoflora. 10 gm of rhizosphere soil was thoroughly mixed with 100 ml sterile water using magnetic stirrer for 15 minutes

in a sterile environment. Serial dilutions were made in an order of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} using sterile distilled water. 1 ml aliquot of each dilutions was added to a sterile Petri plate containing Potato Dextrose Agar medium (PDA) supplemented with 0.075 gm/L ambistryn-S antibiotic. The Petri plates were swirled in clockwise and anticlockwise direction to disperse soil suspension on the medium and incubated for seven days at 28°C in an incubator and the colony growth was monitored. The isolation of each sample was done in triplicates. The colonies of fully-grown fungi were isolated individually and identified following the published literature and identification manuals (Barnett and Hunter, 1972), (Khulbe, 2001), (Raper and Thom, 1949), (Nelson *et al*., 1983), (Onions *et al*., 1981).

3. Screening of isolated pure fungal cultures for the synthesis of 2AP

The pure cultures were screened for the synthesis of 2AP on a synthetic medium proposed by Rungsardthong and Athapol, (2005) (Table 2).

Table 2. Composition of synthetic medium used for 2AP screening (Rungsardthong and Athapol, 2005)

Glucose	15.5 g/l
Putrescine	2.0 g/l
$MgSO_4.7H_2O$	0.5 g/l
NaCl	0.1 g/l
$CaCl_2.2H_2O$	0.1 g/l
Phosphate buffer, pH 7.2	20mM
Vitamin solution	1.0 ml
Biotin	50 mg/l
Ca. Pantothenate	400 mg/l
Inositol	2000 mg/l
Pyridoxine.HCl	400 mg/l
Thiamine.HCl	500 mg/l
Mineral solution	1.0 ml
$MnSO_4.H_2O$	100 mg/l
$ZnSO4.7H_20$	300 mg/l
$CuSO_4.5H_2O$	100 mg/l
$FeCl_3.6H_2O$	250 mg/l
Ammonium molybdate	50 mg/l
H_3BO_3	300 mg/l

For screening, one loop full of the seed culture was transferred to 100 ml synthetic medium in a 500 ml conical flask and kept under agitation in an incubator shaker at 28^0 C, 100 rpm for 72 h. After every 24 h, 10 ml of liquid medium containing growing mycelia was taken out and filtered to remove mycelia. The pH of the filtrate was adjusted at pH 8.0 using sodium bicarbonate and the filtrates were sniffed to detect the presence of 2AP against standard 2AP as a positive control. Among these isolates 30 were found to synthesize 2AP in liquid culture, (Table 2). Fig. 1 shows variety wise percentage of 2AP synthesizing rhizosphere fungi.

4. Extraction and Qualitative detection of 2AP by GC-FID

For extraction and qualitative detection of 2AP, the pure culture of *Aspergillus terreus* was transferred to a conical flask containing 1000 ml synthetic medium and kept on reciprocating shaker at 28 ^{0}C with 100 rpm. After 72 hr of incubation, fungal mycelium was removed by filtration; pH of the filtrate was adjusted to pH 8.0 and transferred into a 1000 ml separating funnel. The volatile contents in the filtrate were extracted twice using 250 ml diethyl ether. The ether was dried over 20 g anhydrous sodium sulphate and condensed using Rota evaporator at 28^{0}C by keeping at 80 rotations per minute. 1 µl of condensed extract was injected to GC (Shimadzu 17 A, Kyoto, Japan) with BP-20 capillary column (SGE, Ringwood, Australia) (30 m x0.32 lm) having flame ionization detector (FID). The injector temperature was kept at 250 ^{0}C and detector was at 260 ^{0}C. The GC oven temperature was held for 2 min at 50 ^{0}C and then ramped to 100 ^{0}C at the rate of 7 ^{0}C /min with a hold of 1 min and then ramped to 250 ^{0}C at the rate of 15 ^{0}C /min with a final hold of 2 min. 2AP peak was identified by matching the GC graph with standard 2AP graph and its retention time (7 min) (Fig.2).

Table 3. Details of rhizosphere fungal isolates

S.No.	Basmati Rice Varieties	No. of isolates	No. of isolates synthesizing 2AP
1	Sharbati Basmati (Moradabad)	20	10
2	Sharbati Basmati (Rampur)	15	4
3	Sharbati Basmati (Pilibhit)	16	8
4	Sharbati Basmati (Nainital)	12	0
5	Golden Basmati	11	1
6	Basmati 11 21 A	11	3
7	Basmati 11 21 B	11	0
8	Basmati Sugandha	10	2
9	Pusa Basmati	12	1
10	Double Chabi	11	4
11	Toda Basmati	15	4
12	Royal Bhog	11	1
13	Dehradun Basmati	16	3
14	Basmati 999	13	5
15	Basmati CH30	14	0
16	Karnal Local	9	1

RESULTS

North India is well known for the cultivation of Basmati rice varieties like Dehradun Basmati and Karnal local are being cultivated since more than 100 years. Therefore for this study Basmati rice varieties were collected from different localities of North India. Total 207 fungal strains were isolated from the rhizosphere of 13 different Basmati rice varieties screened for 2AP detection.

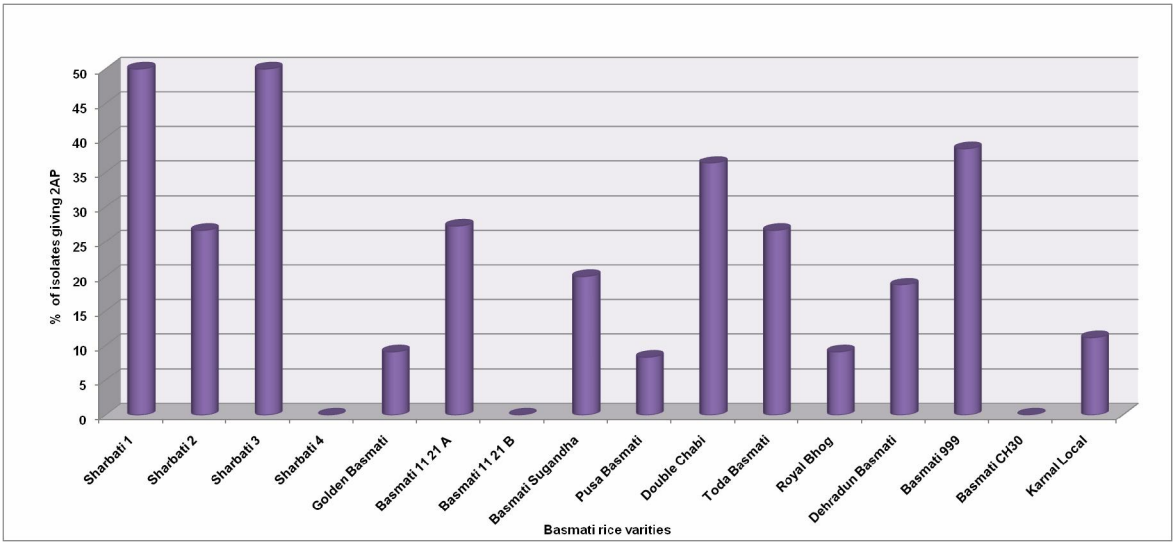

Fig. 1. Graph showes variety wise percentage of 2AP synthesizing rhizosphere fungi

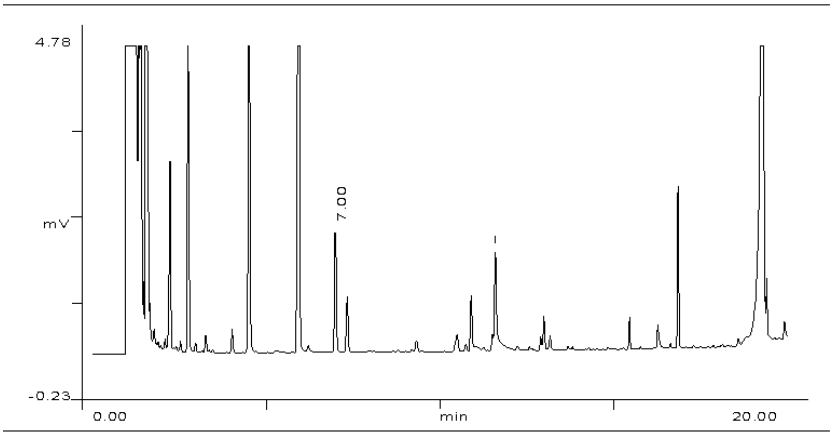

Fig.2. Qualitative analysis of aroma volatiles isolated from *Aspergillus terreus* by GC. Note the 2AP peak marked by arrow

Among 207 isolates 30 were found to synthesize 2AP. When these isolates were screened for 2AP synthesis, it was observed that Sharbati Basmati from Moradabad and Sharbati Basmati from Pilibhit showed the maximum species synthesizing 2AP whereas, basmati 11 21 and Basmati CH30 recorded nil species synthesizing 2AP. Table 3 depicts details of fungal isolates synthesizing 2AP and Fig 1. depicts variety wise percentage of fungal isolates giving 2AP. Among the fungal species synthesizing 2AP, *Aspergillus* species were dominating followed by *Nigrospora* species. Similar observations were recorded by the previous workers. Rungsardthong, (1995) reported *Aspergillus awamori* and *Acremonium nigricans* synthesizing 2AP from the rhizosphere of scented rice varieties. (Nagsuk *et al*., 2003) isolated and identified 2AP in the cultures of *Aspergillus oryzae* TISTR 3088 and *Aspergillus oryzae* TISTR 3232. *Aspergillus terreus* was taken for qualitative analysis of 2AP. 2AP peak was identified by matching the GC graph with standard 2AP graph and its retention time (7 min) (Fig.2).

DISCUSSION

The study highlights the active role of rhizosphere fungi in synthesizing 2AP. Quantification of 2AP and other volatiles synthesized by these fungal species is under study. The study highlights the active role of rhizosphere fungi synthesizing 2AP. This study further opens door to study the plant-fungal metabolism relationship at molecular level.

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