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Short communication

Genetic diversity in *Acorus calamus* L. as revealed by RAPD markers and its relationship with β-asarone content and ploidy level

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ABSTRACT

β-Asarone content in *Acorus calamus* is a paramount issue because it limits the usage of plant for medicinal purpose. In the present study *A. calamus* L. accessions based on RAPD marker, ploidy level and β-asarone content were characterized and correlated on the basis of β-asarone content/ploidy level. Of the 40 random primers used, 6 primers generated polymorphism. Genetic relatedness among accessions evaluated by a similarity matrix based on Dice's coefficient ranged from 0.72 to 0.97. A phenetic dendrogram based on UPGMA analysis grouped accessions into two clusters. *A. calamus* L. accessions were found to be triploid and tetraploid and their β-asarone content was found in two ranges 6.92–8.0% and 73–88%. The study clustered the accessions as per their ploidy level, β-asarone content and geographical locations. This study would have extensive application in quality control of raw materials.

1. Introduction

Acorus calamus L. or 'Sweet Flag' (Araceae) is a reed like semi-aquatic perennial plant with a stout aromatic rhizome having medicinal properties (Agarwal et al., 1956; Motley, 1994). In India it grows in wild in abundance up to 2200 m in Himalayas. The rhizome contains active ingredients possessing insecticidal, antifungal, antibacterial, larvicidal, antitermite, larval and insect repellant properties (Raina et al., 2003; Bertea et al., 2005). In the Ayurvedic system of medicine, the rhizomes are considered to possess antispasmodic, antidiarrhoeic, carminative and anti-helminthic, antidepressant, CNS, anxiolytic properties (Mcgaw et al., 2002; Raina et al., 2003; Bertea et al., 2005). It is used to treat insomnia, melancholia, neurosis, epilepsy, hysteria, loss of memory, remittent fever, rheumatism, toothache, and respiratory ailments (Mcgaw et al., 2002; Mehrotra et al., 2003; Raina et al., 2003).

The essential oil of *Acorus* contains various constituents. The proportion of each chemical constituent of the oil particularly β-asarone varies between the varieties of *A. calamus* and corresponds to the ploidy level (Mcgaw et al., 2002). From a karyotypic point of view, sweet flag includes four ploidy levels: diploid (2x = 24), triploid (3x = 36), tetraploid (4x = 48) and hexaploid (6x = 72) (Bertea et al., 2005). Diploid karyotypes grow in North America and in parts of Asia and are characterized by the absence of β-asarone, whereas European, North American and Kashmiri triploid karyotypes contain 3–19% of β-asarone. The Indian, Indonesian and Taiwan tetraploid karyotypes contain up to 96% of β-asarone (Mcgaw et al., 2002; Bertea et al., 2005), whereas tetraploid of Far East Russia are characterized by 10–40% β-asarone (Raina et al., 2003).

β-Asarone (z)-1,2,4-trimethoxy-5-prop-1-enyl-benzene is a toxicant causing chromosomal aberrations, mutations and cancer (Goggelman and Schimmer, 1983; Abel, 1987). Because of varying β-asarone content, precise identification of *A. calamus* chemotypes is a prerequisite for commercial application. Conventionally, identification of herbals was based on morphological, anatomical and chemical analysis but these could be influenced by environmental factors. Identification of DNA markers that can correlate DNA fingerprinting data with quantity of selected phytochemical marker associated with that particular class of plant would have extensive application in quality control of raw materials. Among various molecular techniques, RAPD is a simple, largely automatable technique require only small amount of DNA and can be performed without the use of radioactivity (Williams et al., 1990). This technique is an efficient and inexpensive method of generating molecular data has been employed in many taxonomic and phylogenetic studies (Keil and Griffin, 1994; Khan et al., 2000). Although RAPD technique is outdated technique, still it offers several advantages in species or taxon or chemotype identification. It was also used in prediction of phytochemicals in plants (Chen et al., 2009).

Since the correlation of RAPD data with β-asarone content of these accessions and their ploidy level would be useful for plant breeding, quality control, intellectual property rights and eventually

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for pharmacological studies, the present study was made to characterize *A. calamus* L. accessions based on RAPD marker, ploidy level and β-asarone content and correlate them on the basis of β-asarone content/ploidy level.

2. Materials and methods

2.1. Plant material

Twenty-three accessions of *A. calamus* L. were from four states in northern and north-east India namely Uttarakhand (UK), Himachal Pradesh (HP), Jammu & Kashmir (J&K) and Manipur (MN) representing four agro-ecological regions (Table 1). They were planted in an herbal garden and conserved through clonal propagation.

2.2. β-Asarone content analysis

The essential oil was obtained from aerial parts of naturally grown plants by hydro-distillation using a Clevenger-type apparatus. Triplicate distillations were performed in succession for each sample. The oil samples were stored at 4 °C until used for chemical analysis. Gas chromatography (GC) analysis of the oil was carried out on NUCON Gas Chromatograph apparatus, fitted with a fused silica capillary column coated with FFAP and helium as carrier gas. The compounds were identified by comparison of their relative retention times with those of known compounds run under similar conditions and by enrichment technique. GC–MS was recorded on QP-2000 Shimadzu Model fitted with BP-10 column. The GC oven temperature was programmed as follows: initial temperature 90 °C/2 min to 220 °C with rise at the rate of 7 °C/min to 220 °C. The carrier gas was helium with FID detector. The samples were injected using split sampling method, ratio 1:50. The content of β-asarone (retention time 18.6 min) in the relative percentage was computed by the normalization method from GC peak areas. All 23 accessions were analyzed for β-asarone content.

2.3. Ploidy analysis

For cytological studies growing root tips pretreated with a saturated solution of p-dichlorobenzene for 4 h at 20 °C. Following washing is distilled water; the material was fixed in mixture of acetic acid and ethanol (1:3) and exposed to a chilling treatment (4 °C) for 4 h. Subsequently the material was transferred to 70% alcohol for further use. For tissue staining orecein dye was used (orecein 1%, 1N HCl, 18:1). Squash preparations were made in acetocarmine and chromosomal analysis was done at the metaphase/anaphase stage. Data on ploidy level was computed from observations of about 100 cells per sample. All 23 accessions were analyzed for ploidy level.

2.4. RAPD analysis

Young and fresh leaves (0.2 g) of randomly selected plants of various accessions were collected and used for genomic DNA extraction (Ahmad et al., 2004). Forty decamer primers were initially screened to generate RAPD profiles. RAPD profiles were generated as described by Ahmad et al., 2006. A control PCR tube containing all components, but no genomic DNA was run with each primer to check for contamination. All the PCR results were tested for reproducibility by at least three times. Bands that did not show fidelity were eliminated for statistical analysis.

Discriminating power (Dj) of each primer, i.e., the probability that the two randomly chosen accessions from the sample of 23 accessions have different banding pattern and, thus, are distinguishable from one another, was estimated (Tessier et al., 1999). Genetic diversity was estimated by Shannon index (Lewontin, 1972). To investigate phenetic relationships among accessions, the binary matrix was used to cluster individuals using procedure of NTSYS-PC2.1 (Rohlf, 1993). A dendrogram was constructed based on Dice coefficient's similarity data applying the unweighted pair group method (UPGMA). The robustness and validity of clustering pattern was tested by Bootstrap analyses of 1000 bootstrap samples using the software *WINBOOT* (Yap and Nelson, 1996).

3. Results and discussion

3.1. β-Asarone content and ploidy analysis

All 23 accessions analyzed for chemical spectrum and ploidy level. Accessions AC8 and AC10 belonging to Kashmir (J&K) and AC4, AC6 and AC7 belonging to Manipur were found to be triploids

Table 1
Acorus calamus accessions collected from different geographical locations in India with their ploidy level and β-asarone content (%).

Sr. no.	Accession code no.	Place of collection	Geograpical locations	Ploidy level	β-Asarone content (%)
1	AC1	Palampur (HP)	32°N 76°E	Tetraploid	88.0
2	AC2	Mandi (HP)	31°N 76°E	Tetraploid	87.0
3	AC3	Palampur (HP)	32°N 76°E	Tetraploid	80.0
4	AC4	Manipur (MN)	25°N 94°E	Triploid	7.0
5	AC5	Shamshi (HP)	31°N 77°E	Tetraploid	78.0
6	AC6	Manipur (MN)	25°N 94°E	Triploid	7.34
7	AC7	Manipur (MN)	25°N 94°E	Triploid	7.85
8	AC8	Narkara, Kashmir (J&K)	34°N 74°E	Triploid	8.0
9	AC9	NGC Kullu (HP)	31°N 77°E	Tetraploid	76.0
10	AC10	Kakpura, Kashmir (J&K)	34°N 74°E	Triploid	6.92
11	AC11	TP Batote, Jammu (J&K)	32°N 74°E	Tetraploid	76.0
12	AC12	Rani Sidhpur, Jammu (J&K)	32°N 74°E	Tetraploid	82.0
13	AC13	Hirabagh, Jammu (J&K)	32°N 74°E	Tetraploid	78.0
14	AC14	Chimbelhar (HP)	32°N 76°E	Tetraploid	82.4
15	AC15	Tikaphata (HP)	31°N 77°E	Tetraploid	84.0
16	AC16	Tipovan (HP)	34°N 77°E	Tetraploid	87.2
17	AC17	Manali (HP)	32°N 77°E	Tetraploid	76.0
18	AC18	Manali (HP)	32°N 77°E	Tetraploid	73.0
19	AC19	Manali (HP)	32°N 77°E	Tetraploid	81.0
20	AC20	Manali (HP)	32°N 77°E	Tetraploid	84.0
21	AC21	Manali (HP)	32°N 77°E	Tetraploid	80.0
22	AC22	Barotiwala (HP)	31°N 77°E	Tetraploid	86.0
23	AC23	Dehradun (UK)	30°N 78°E	Tetraploid	86.0

HP: Himachal Pradesh; UK: Uttarakhand; MN: Manipur; J&K: Jammu & Kashmir.