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Research Article

Protective role of antioxidant enzymes in chickpea (*Cicer arietinum* L.) genotypes under high temperature stress

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ABSTRACT

Four chickpea genotypes differing in their sensitivity to high temperature stress were taken and grown in growth chambers in the phytotron facility of IARI, New Delhi. The plants were maintained at 18/23°C (control) and 25/35°C (temperature stress) night/day temperature after maximum tillering. In all chickpea genotypes high temperature stress increased membrane injury index (MII), activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX), malic acid and slightly decreased the activity of glutathione reductase (GR), relative water content (RWC), chlorophyll and carotenoid contents. Under the HT stress, the tolerant genotypes Pusa-1103 and BGD-72 exhibited higher RWC, chlorophyll and carotenoid, activity of SOD, APX, GR and, and less decrease in MII as compared to susceptible genotypes Pusa-256 and RSG-991. Antioxidant enzymes showed positive correlation (*r*) with chlorophyll content, RWC and negative with MII under high temperature stress. From the results it is apparent that the antioxidant defence mechanism plays an important role in heat stress tolerance of chickpea genotypes.

Keywords: High temperature stress; Phytotron; Membrane injury index; Antioxidant enzymes; Chlorophyll contents; Relative water content.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the most important cool-season legumes grown extensively throughout the world, particularly in West and South Asia and North African countries. It has been grown in semiarid regions of the world for hundreds of years. The major chickpea producers India, Pakistan and Turkey contribute 67.0, 9.5 and 6.7%, respectively to the world harvest [1]. India predominates in chickpea supply and it has the distinction of being the largest producer and consumer in the world. The important chickpea growing states of India are Madhya Pradesh, Punjab, Haryana, Rajasthan, Uttar Pradesh, Bihar, Gujarat, Maharashtra, Karnataka and Andra Pradesh. Latest estimate for 2007-08 indicate that the production of pulses in the country is 16.64 million tonnes from an area of 26.15 million hectares. Chickpea is the most important pulse recorded production of 6.43 million tonnes during 2007-08. Inspite of having largest area under chickpea in the world India's position in average productivity is yet to see major breakthrough to meet the per capita availability of 50 g pulses/day to alleviate proteins energy malnutrition.

In northern India, however, late planting of chickpea is done after harvest of rice, early potato or cotton. Such late sown chickpea crop experiences high temperature at the end of the cropping season. This high temperature at the end of cropping season

leads to problem of poor biomass and forced maturity [2]. The Inter-Governmental Panel on Climate Change (IPCC) of the United Nations in its recent report has confirmed the global warming trends, and projected that the globally averaged temperature of the air above the earth's surface would rise by 1.4-5.8 ^{o}C over the next 100 years [3].

This high temperature stress leads to the production of reactive oxygen species (ROS) which damages the plant cellular and subcellular system. However, plants protect its systems from cytoxic effects of the reactive oxygen species using antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, ascorbic acid and carotenoides [4]. Use of ion leakage and relative water content as simple indices for screening genotypes against heat and drought stress in chickpea and wheat has been suggested by many workers [5, 6].

The present work was conducted to study the effect of high temperature stress on antioxidant enzymes in chickpea genotypes.

2. MATERIALS AND METHODS

2.1. Selection and propagation of genotypes

The experiment was conducted at phytotron facility of I.A.R.I., New Delhi, with four chickpea (*Cicer arietinum* L) genotypes differing in sensitivity to high temperature (HT) stress *i.e*., Pusa-1103 and BGD-72 (HT tolerant), Pusa-256 and RSG-991 (HT susceptible). Seeds of these four selected genotypes were collected from Genetics Division, I.A.R.I., New Delhi. They were treated with *Mesorhizhobium ciceri* SPG strain and were sown in earthen pots (20×30 cm^2) containing mixture of soil, sand and farmyard manures (FYM) in ratio of 3:1:1. Recommended dose of nitrogen, phosphorus and potassium fertilizers were applied. Seeds were sown in each pot by dibbling method at 2 cm depth. Thinning was done at 10 days after sowing, and five plants were retained in each pot.

2.2. High temperature treatment

Chickpea plants were exposed to temperature stress by covering them with polyvinyl chloride sheets (Capri Hans, sunflex 0.15 mm thickness and transmittance 85%) mounted on wooden structures of size 3 x 2 x 2 m. The wooden polythene chambers were kept 10 cm above the ground for circulation of air and to control the humidity inside the chamber. Thermometer was placed inside the poly cover and the level of temperature was recorded regularly. The temperature inside the poly cover was 6.1 ^{o}C higher than the ambient temperature. Twenty pots from each of the four genotypes were shifted inside the polycovers at the 78 DAS to expose plants to temperature stress. Thirty seven days after temperature stress, all the pots were taken out and kept under the normal environment (ambient temperature), and the physiobiochemical observations were recorded.

2.3. Physiobiochemical estimation

Relative water content in leaves was estimated according to the method described by [7]. Membrane injury index was estimated from all genotypes in three random replicates as suggested by [6]. Chlorophyll content was determined according to [8] and carotenoid content was estimated according to [9].

Enzyme extract for superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase was prepared by first freezing the weighed amount of leaf samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM ascorbic acid). Brie was passed through 4 layers of cheese cloth and filtrate was centrifuged for 20 min at 15000 rpm at 4 ^{o}C and the supernatant was used as enzyme.

Superoxide dismutase activity was estimated by recording the enzyme induced decrease in absorbance of formazone made by nitro-blue tetrazolium with superoxide radicals [10].

Ascorbate peroxidase activity was estimated by observing the decrease in absorbance due to ascorbic acid at 290 nm and the Glutathione reductase activity was assayed by recording the increase in absorbance in the presence of oxidized glutathione and DTNB (5, 5-dithiobis-2-nitrobenzoic acid [11].

2.4. Statistical analysis

Pearson-product-moment correlation coefficient (*r*) between various antioxidant enzymes and total chlorophyll content, relative water content and membrane injury index were computed according to [12].

3. RESULTS AND DISCUSSION

3.1. Relative water content

The analysis of data (Table 1) showed that with the increase in temperature stress there was progressive decrease in the RWC of flag leaves. Under ambient temperature, the RWC was higher in BGD-72 and Pusa-1103 and less in Pusa-256 and RSG-991. However, under high temperature (HT) treatment Pusa-1103 and BGD-72 showed significantly higher RWC. Average decline in RWC under high temperature stress was 14.21%, Pusa-1103 showed lowest per cent decline (6.0%), while RSG-991 showed highest decline (18.99%) in RWC. Significant differences were also obtained between treatment and genotypic interaction.

3.2. Membrane injury index

The membrane injury Index (MII) decreased under heat stress in all the genotypes (Table 2.). However, under ambient temperature (AT) the MII was lowest in Pusa-1103 (31.61%) closely followed by BGD-72, and highest in Pusa-256. Under HT stress, the Pusa-1103 showed lowest MII and RSG-991 exhibited highest MII (46.47%). The average increase in MII under high temperature stress was 19.33%, Pusa-1103 showed lowest increase (9.80%), while RSG-991 showed highest increase (31.74%) in MII. The interaction between treatment and genotypes was also significant.

Table 1. Effect of high temperature stress on relative water content in (%) chickpea genotypes.

Genotypes	Control	Treatment	Genotypic mean	Percent decrease
Pusa 1103	80.21	75.49	80.35	6.00
BGD 72	83.67	71.40	77.53	14.79
Pusa 256	78.53	65.24	71.88	17.06
RSG 991	78.34	63.57	70.95	18.99
Mean	80.19	68.93		14.21
CD at 5%				
Treatment(T)	1.92			
Genotypes(G)	2.71			
T x G	3.83			

Table 2. Effect of high temperature stress on membrane injury index (%) in chickpea genotypes.

Genotypes	Control	Treatment	Genotypic mean	Percent decrease
Pusa 1103	31.61	34.67	33.14	9.80
BGD 72	34.21	39.87	37.04	16.68
Pusa 256	36.28	43.16	39.72	19.10
RSG 991	35.32	46.47	40.90	31.74
Mean	34.36	41.04		19.33
CD at 5%				
Treatment (T)	1.38			
Genotypes(G)	0.97			
T x G	1.95			

3.3. Photosynthetic pigments

Data on chlorophyll 'a', chlorophyll 'b', total chlorophyll, total carotenoid, chlorophyll a/b ratio are reported in (Figs. 1-5). Chlorophyll 'a', chlorophyll 'b', total chlorophyll, total carotenoid and chlorophyll/carotenoid ratio in leaves of chickpea genotypes were higher under AT condition, and a significant decline was observed for all the above parameters under HT condition. Pusa-1103 followed by BGD-72 exhibited significantly higher chlorophyll 'a', chlorophyll 'b', total chlorophyll, and chlorophyll a/b ratio compared to Pusa-256 and RSG-991 under both the conditions.

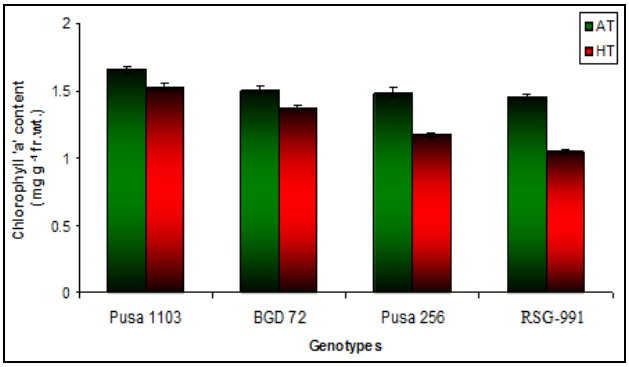

Figure 1. Effect of high temperature stress on chlorophyll 'a' content on chickpea genotypes. Vertical bars show ± S.E of mean. Data for treatments (T) and genotypes (G) and T x G interactions were significant (P = 0.05).

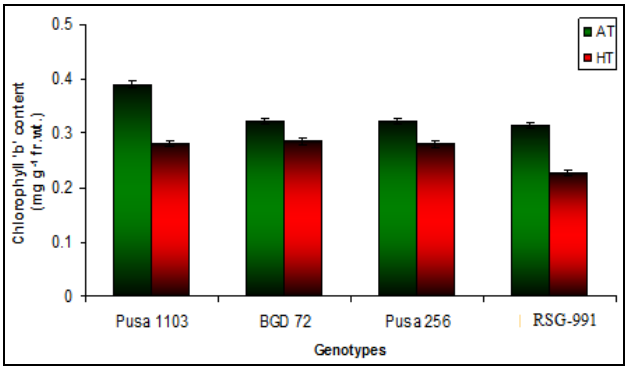

Figure 2. Effect of high temperature stress on chlorophyll 'b' content on chickpea genotypes. Vertical bars show ± S.E of mean. Data for treatments (T) and genotypes (G) and T x G interactions were significant (P = 0.05).

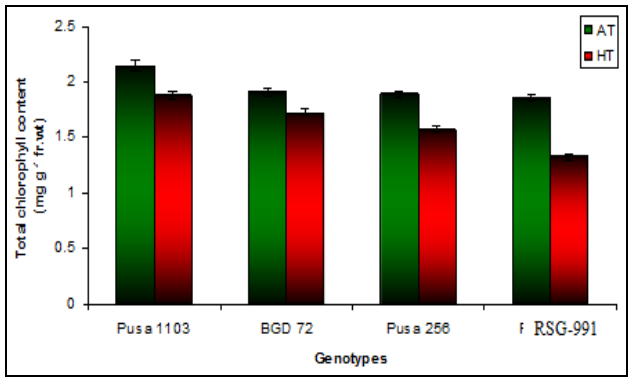

Figure 3. Effect of high temperature stress on total chlorophyll content on chickpea genotypes. Vertical bars show ± S.E of mean. Data for treatments (T) and genotypes (G) and T x G interactions were significant (P = 0.05).

Total carotenoid content was significantly higher in Pusa-256 under AT condition along with Pusa-1103 and BGD-72. There was a general decline of 15.87% in carotenoid content in all genotypes under HT condition. Under HT condition RSG-991 showed higher reduction, while Pusa-1103 maintained comparatively higher carotenoid content. Under AT condition the chlorophyll/carotenoid ratio was higher in BGD-72 and Pusa-1103 closely followed by RSG-991 and lowest in Pusa-256. Under HT treatment, there was a non significant reduction in chlorophyll/carotenoid ratio, and among genotypes Pusa-1103 possessed highest chlorophyll/ carote-noid ratio.

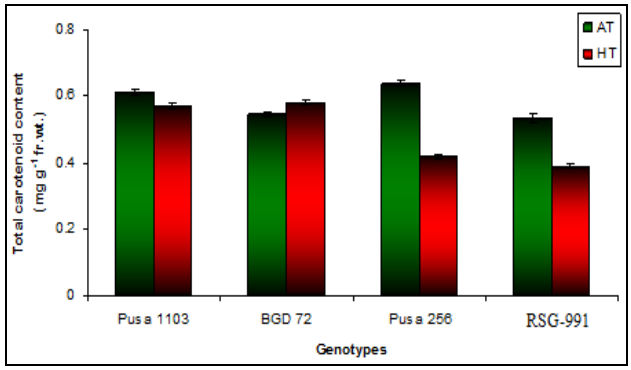

Figure 4. Effect of high temperature stress on total carotenoid content on chickpea genotypes. Vertical bars show ± S.E of mean. Data for treatments (T) and genotypes (G) and T x G interactions were significant (P = 0.05).

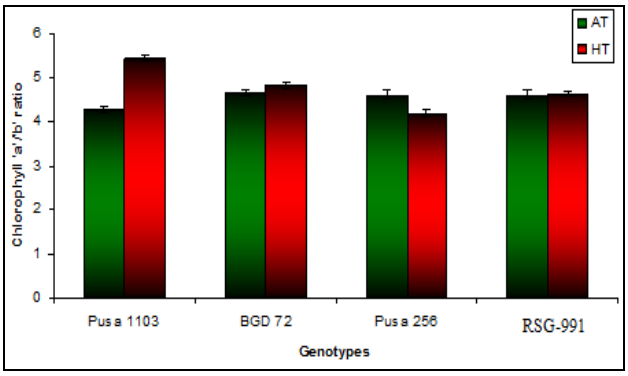

Figure 5. Effect of high temperature stress on chlorophyll 'a'/'b' content on chickpea genotypes. Vertical bars show ± S.E of mean. Data for treatments (T) and genotypes (G) and T x G interactions were significant ($P = 0.05$).

3.4. Antioxidant enzyme activities

Superoxide dismutase (SOD) activity showed significant increase under HT compared to AT condition (Table 3). Among the genotypes, BGD-72 showed significantly higher SOD activity followed by Pusa-1103 and Pusa-256. RSG-991 exhibited the lowest activity under HT condition. SOD activity increased by two folds in BGD-72 and by 5% in RSG-991. Significant interaction was observed between treatment and genotypes.

APX increased significantly under HT condition compared to AT (Table 4). Under both the conditions Pusa-1103 possessed higher APX activity, while RSG-991 showed the lowest activity. BGD-72 and Pusa-256 exhibited moderate activity under both the conditions.

Table 3. Effect of high temperature stress on superoxide dismutase activity (units $min^{-1}mg^{-1}$ protein) in chickpea genotypes.

Genotypes	Control	Treatment	Genotypic mean	Percent increase
Pusa 1103	3.80	5.92	4.86	55.9
BGD 72	3.82	6.20	5.01	62.4
Pusa 256	2.72	4.75	3.74	74.7
RSG 991	2.92	3.07	2.99	5.1
Mean	3.31	4.98		50.4
CD at 5%				
Treatment (T)	0.12			
Genotype (G)	0.17			
T x G	0.25			

Table 4. Effect of high temperature stress on ascorbate peroxidase activity (µmol ascorbate oxidized $min^{-1}mg^{-1}$ protein) in chickpea genotypes.

Genotypes	Control	Treatment	Genotypic mean	Percent increase
Pusa 1103	6.33	11.46	8.89	81.0
BGD 72	6.16	9.22	7.69	49.8
Pusa 256	6.02	7.20	6.61	19.7
RSG 991	5.97	6.59	6.28	10.4
Mean	6.12	8.62		40.8
CD at 5%				
Treatment (T)	0.25			
Genotype (G)	0.36			
T x G	0.50			

Table 5. Effect of high temperature stress on glutathione reductase activity (Δ A $_{412}$ min^{-1} mg^{-1} protein) in chickpea genotypes.

Genotypes	Control	Treatment	Genotypic mean	Percent increase
Pusa 1103	5.43	4.63	5.03	14.8
BGD 72	5.29	4.06	4.67	23.3
Pusa 256	4.76	3.44	4.10	27.6
RSG 991	4.12	1.91	3.02	53.8
Mean	4.90	3.51		28.4
CD at 5%				
Treatment (T)	0.19			
Genotype (G)	0.27			
T x G	0.37			

Table 6. Correlation coefficient (*r*) between various enzymes and chlorophyll content, relative water content and membrane injury index in chickpea genotypes.

Antioxidant enzymes	Chlorophyll content		Membrane injury index		Relative water content	
	AT	HT	AT	HT	AT	HT
SOD	0.559*	0.949**	- 0.714**	-0.886**	0.614*	0.855**
APX	0.404	0.884**	0.342	-0.946**	0.052	0.940**
GR	0.092	0.956**	0.473	-0.899**	-0.433	0.836**

* Significant at 5%; ** Significant at 1%.

Data on glutathione reductase (GR) activity is reported in (Table 5). GR activity decreased significantly under HT condition. Under AT condition Pusa-1103 showed higher activity followed by BGD-72. Under HT condition also Pusa-1103 and BGD-72 maintained a higher GR activity, while RSG-991 exhibited a greater decline. Significant interaction was observed between treatment and genotypes for GR activity.

3.5. Correlation studies

Results on Pearson-product- moment correlation coefficient (r) between various antioxidant enzymes, and total chlorophyll content, relative water content and membrane injury index are reported in (Table 6). The results revealed that under high temperature condition there exist a significant positive correlation between antioxidant enzymes and chlorophyll content and RWC, and significant negative correlation with MII. However under ambient temperature non significant correlation was observed between antioxidant enzymes and other parameters. Among the three antioxidant enzymes, the glutathione reductase showed significantly higher correlation (r) with other three physiological traits under high temperature condition.

4. DISCUSSION

Plants experience high temperature in many different ways and adaptation or acclimation to high temperature occurs over different levels of plant organization [13]. In this study the BGD-72 and Pusa-1103 maintained higher RWC, chlorophyll, carotenoid contents and lower MII under high temperature condition than Pusa-256 and RSG-991. This shows that the tolerant genotype Pusa-1103 had a greater water retention capacity under HT stress. Due to high temperature induced higher transpiration situation similar to water stress is created and RWC becomes important under heat stress. Heat stress injury involves water deficit and cell turgor loss [14]. Maintenance of favorable water status is essential for plant's tolerance to heat stress [15, 16].

Heat stress induced decrease membrane stability has been reported in faba bean leaf discs [17, 18] also reported that the tolerant genotypes possess lower membrane injury index and high RWC, which enable them to maintain better metabolic activities.

Leaf photosynthetic pigment content (chlorophylls and carotenoids) and pigment ratios, such as Chl a/b is good indicators for stress detection and tolerance [18]. Chlorophyll 'a', chlorophyll 'b', total chlorophyll and total carotenoid contents in leaves of chickpea genotypes decreased, while an increase was observed in Chl a/b ratio under HT condition compared to AT condition. Camejo et al. [19] also showed increase in the chlorophyll a/b ratio in stressed Nagcarlang tomato plants, suggesting that these relationships could be used as an indicator of tolerance and physiological status of the plants under stress condition.

Carotenoid protects the photosynthetic systems against singlet oxygen and also plays a pivotal role in thermal dispersion of excess excitation energy [20-23]. Higher carotenoid content in tolerant genotypes Pusa-1103 and BGD-72 signifies their tolerance capacity.

Tolerance to high temperature stress in crop plants has been reported to be associated with an increase in antioxidant enzymes activity [24-27] reported significant increase in SOD activity under temperature stress in wheat genotypes, and a greater increase in tolerant genotype C 306, while the susceptible genotypes showed lower activity. In this study the chickpea tolerant genotypes BGD-72 and Pusa-1103 exhibited higher activity of SOD, APX and GR compared to susceptible genotypes. This shows that the tolerant genotypes combated the ROS by maintaining efficient antioxidant mechanism. Sairam et al. [4] and Almeselmani et al. [28] also reported similar results in late sown heat tolerant wheat varieties. Higher activity of various antioxidative enzymes in temperature tolerant genotypes of various crop species has also been reported by various workers [4, 28-30].

Study of correlation coefficient between various antioxidant enzymes with chlorophyll content, relative water content and membrane injury index at high temperature revealed a positive correlation with chlorophyll content, relative water content and negative correlation with membrane injury index. Almeselmani et al. [28] also reported a significant positive correlation between chlorophyll content and antioxidant enzymes and negative correlation between membrane injury index and antioxidant at high temperature stress condition.

5. CONCLUSION

From the foregoing discussion it is clear that exposure of chickpea genotypes to high temperature stress for a medium duration of thirty seven days i.e., 78 to 115 DAS resulted in increase in the activity of superoxide dismutase, ascorbate peroxidase and glutathione reductase. The temperature tolerant genotypes Pusa-1103 and BGD-72 exhibited a comparatively higher superoxide dismutase, ascorbate peroxidase and slight decline in glutathione reductase compared to susceptible genotypes Pusa-256 and RSG-991. Efficient antioxidant enzymes status in tolerant genotypes under high temperature condition reflected in lower membrane injury index, higher relative water content, chlorophyll and carotenoid content compared to susceptible genotypes Pusa-256 and RSG-991. Hence selection of genotype based on these criteria may help in evolving chickpea genotypes tolerant to high temperature stress with better yield.

TRANSPARENCY DECLARATION

The author declares that there is no conflict of interests.

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Research Article

Study of insecticidal properties of *Trachyspermum ammi* and *Mentha arvensis* essential oils against *Sitophilus zeamais* L. (Coleoptera: Curculionidae)

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ABSTRACT

Trachyspermum ammi and *Mentha arvensis* essential oils have been evaluated for repellent, insecticidal and oviposition inhibitory activities in maize weevil, *Sitophilus zeamais*. In repellency assay, *T. ammi* and *M. arvensis* essential oils showed repellent activity against *S. zeamais* adults. These essential oils caused mortality in *S. zeamais* adults when applied by fumigation and contact methods. In fumigation toxicity assay, median lethal concentrations (LC_{50}) of *T. ammi* and *M. arvensis* essential oils were 0.385 and 0.323 µl/cm^3, and 0.274 and 0.214 µl/cm^3 air after 24 and 48 h exposure of *S. zeamais* adults respectively. In contact toxicity assay, median lethal concentrations (LC_{50}) of *T. ammi* and *M. arvensis* essential oils were found 0.317 and 0. 278 µl/cm^2, and 0.204 and 0.169 area after 24 and 48 h exposure of *S. zeamais* adults respectively. Essential oils of *T. ammi* and *M. arvensis* oils inhibited progeny production by inhibiting oviposition in *S. zeamais* adults when exposed to sub-lethal concentrations. This study concludes that *T. ammi* and *M. arvensis* oil can be used as alternative in management of stored-grain insects.

Keywords: *Trachyspermum ammi*; *Mentha arvensis*; Essential oil; *Sitophilus zeamais*.

1. INTRODUCTION

With the beginning of agricultural practices and storage of food grains as a safeguard against poor harvests and famine, insects also started damaging stored grains both qualitatively and quantitatively. Several synthetic insecticides have been developed during recent past and applied to protect stored grains from harmful insects. The excessive and continuous use of these synthetic insecticides has increased the risk of ozone depletion, neurotoxicity, carcinogenicity, terato-genicity and mutagenic effects among non-target species and cross-resistance and multi resistance in insects [1-4]. This has raised public and environmental issues diverting attention towards other alternatives sources of stored-grain insect pest management. In this regard, use of plant derived products especially essential oils come into practice. Essential oils, a group of plant origin product, are highly volatile and non-persistent. Some of these exhibit adulticidal, larvicidal and antifeedant activity, oviposition inhibitory activities, capacity to delay development and adult emergence [5-14]. Among 17,500 aromatic plant species of Alliaceae,

Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Myrtaceae, Piperaceae, Poaceae, Rutaceae and Zingiberaceae families, only 3,000 essential oils have been known and 10% have commercial importance in cosmetic, food and pharmaceutical industries [15]. These are complex, secondary metabolites characterized by a strong odour and low density [16]. These oils are mixtures of 20 to 60 compounds of different chemical nature in different concentrations. Each essential oil is characterized by a specific essence due to two or three major components present in fairly high concentrations (20 to 70%). The biological activities of essential oils depend on their chemical composition, which, in turn, varies with plant parts used for extraction, extraction method, plant phenological stage, harvesting season, plant age, genotype of the plant, soil nature and environmental conditions [17, 18].

Trachyspermum ammi (Family: Apiaceae) commonly known as Ajwain is grown in large scale in Rajasthan, Gujarat and Andhra Pradesh and in smaller scale in Uttar Pradesh, Punjab, Bihar, Madhya Pradesh, Tamil Nadu, West Bengal and Karnataka [19, 20]. The umbels of the plant mature and produce small and grey coloured seeds with bitter taste. The essential oil can be obtained from seeds by hydrodistillation using a Clevenger apparatus. *T. ammi* seeds produce 2.5-5% essential oil [21]. The principal constituents responsible for typical flavour of ajwain seed essential oil are thymol and carvacrol. *T. ammi* seed essential oil contains 27 compounds, of which thymol (40%) is present in the largest amounts. Other components are p-cymene (15.6%) and γ-terpinene (11.9%) whereas β-pinene (4%), limonene (4%), carvacrol (5%), camphene and myrcene. *Mentha arvensis* (Family: Lamiaceae), commonly known as corn-mint, menthol mint or Japanese mint is cultivated commercially in many parts of the world in tropical and subtropical climates. The green leaves of *Mentha arvensis* produce 0.30-0.40% yellow coloured volatile oil by hydrodistillation method. Chemical analysis reveals menthone (29.41%), menthol (21.33%), isomenthone (10.80%), eucalyptol (6.91%), neo-menthol (4.70%), *cis*-piperitone oxide (3.62%), linalool (2.20%), thymol (1.60%), dl-limonene (1.47%) and α-phellandrene (3.20%) as major constituents [22].

The maize weevil, *Sitophilus zeamais* (Coleptera: Curculionidae) is a major pest of maize in humid tropical areas of the world where maize is grown [23]. *S. zeamais* is an internal feeder of grains and attacks both standing crops and stored cereal commodities including wheat, rice, sorghum, oats, barley, rye, buckwheat, peas and cottonseed. Females cause damage by boring into the kernel and laying eggs. The larvae and pupae eat the inner parts of the kernel, resulting in a damaged kernel and reduced grain weight [24]. Apart from weight losses, the feeding damage caused by weevils leads to severe reductions in nutritive and economic values, reduced seed viability, as well as contamination by chemical excretions (silk) and insect fragments [25]. The infestation also elevates temperature and moisture content in the stored grain mass, which can lead to mold growth, including toxigenic species such as *Aspergillus flavus* [26]. *S. zeamais* cause extensive losses in quality and quantity of the grain in the field as well as in storage [27]. In the present study, essential oils of *T. ammi* and *M. arvensis* essential oils have been evaluated for their repellent, insecticidal and antiovipositional activities against maize weevil, *S. zeamais*.

2. MATERIALS AND METHOD

2.1. Essential oils

Seeds of *T. ammi* were purchased from the local market of Gorakhpur. Green leaves of *M. arvensis* were collected from local field of Gorakhpur. Essential oils were isolated by hydrodistilation of *T. ammi* seeds and *M. arvensis* leaves for 4 hours in Clevenger apparatus. Essential oils were kept in Eppendorff tubes at 4^0C till further use.

2.2. Insects

Maize weevil, *S. zeamais* was used to determine the insecticide nature of *T. ammi* and *M. arvensis* essential oils. The insects were reared on whole maize grain in the laboratory at 30 ± 4^0C, 75±5% RH, and photoperiod of 10:14 (L:D) hours.

2.3. Repellent activity

Repellency assay was performed in glass petri dishes (diameter 8.5 cm, height 1.2 cm). Test

solutions of different dilutions (0.2, 0.4, 0.8 and 1.6% vol:vol) of *T. ammi* and *M. arvensis* essential oils were prepared in acetone. Whatman filter papers were cut into two halves and each test solution was applied to filter paper half as uniform as possible using micropipette. The other half of the filter paper was treated with acetone only. Essential oil treated and acetone treated halves were dried to evaporate the acetone completely. Both treated and untreated halves were then attached with cellophane tape in a manner so that seepage of the test samples from one half to other half can be avoided and placed at the bottom in each petri dish. Forty *S. zeamais* adults were released at the centre of the filter paper disc and the petri dish was covered and kept in dark. Six replicates were set for each concentration of essential oil. After 4 hours of treatment, number of adults in treated and untreated halves was counted.

Percent repellency (PR) was calculated using formula: PR = (C-T)/(C+T) ×100, C = number of insects in the untreated halves and T = number of insect in treated halves

Preference index (PI) was calculated using the following formula: PI = (percentage of insects in treated halves - percentage of insects in untreated halves)/ (percentage of insects in treated halves + percentage of insects in untreated halves). PI values between - 1.0 and - 0.1 indicate repellant essential oil, - 0.1 to + 0.1 neutral essential oil and + 0.1 to + 1.0 attractant essential oil.

2.4. Fumigant toxicity

Formulations of *T. ammi* and *M. arvensis* essential oils were made by using acetone as solvent. Ten adults taken from the laboratory culture were placed with 2 g of wheat grains in glass petri dish (diameter 8.5 cm, height 1.2 cm). Filter paper strip (2 cm diameter) was treated with essential oil formulations and left for two minutes for evaporation of acetone. Treated filter paper was pasted on the undercover of petri dish, air tightened with parafilm and kept in dark in conditions applied for rearing of insect. Six replicates were set for each concentration of essential oil and control. After 24 and 48 hours of fumigation, mortality in adults was recorded.

2.5. Contact toxicity

Formulations of *T. ammi* and *M. arvensis* essential oils were made in acetone, applied on bottom surface of glass petri dish (diameter 8.5 cm, height 1.2 cm) and left for two minutes for evaporation of acetone. Ten adults taken from the laboratory culture were released at the centre of petri dish, covered and kept in dark in conditions applied for rearing of insect. After 24 and 48 hours of fumigation, mortality was recorded.

2.6. Oviposition inhibitory effect

Ten *S. zeamais* adults of mixed sex were fumigated with sublethal concentrations viz. 40% and 80% of 24-h LC_{50} and 48-h LC_{50} of *T. ammi* and *M. arvensis* essential oils for 24 h and 48 h respectively and reared on wheat grain in a 250 ml plastic box for 10 days. After 45 days, adults were discarded and number of F_1 progeny was counted. Six replicates were set for each concentration of essential oils and control.

2.7. Data analysis

Median lethal concentration (LC_{50}) was calculated using POLO programme [28]. Analysis of variance (ANOVA) and correlation and linear regression analysis were conducted to define concentration-response relationship [29].

3. RESULTS

3.1. Repellent activity

Repellency was 74.33, 98.33 and 100% at 0.2, 0.4 and 0.8% concentrations of *T. ammi* essential oil respectively (Table 1). Preference Index (PI) was -0.74, -0.98 and 1.0 at 0.2, 0.4 and 0.8% concentrations of *T. ammi* essential oil respectively (Table 1). *T. ammi* essential oil showed significant ($F = 238.74$, $P<0.01$) repellency against *S. zeamais* adults. Repellency was 78.66, 94.83 and 100% at 0.2, 0.4 and 0.8% concentrations of *M. arvensis* essential oil respectively (Table 1). Preference Index (PI) was -0.78, -0.94 and 1.0 at 0.2, 0.4 and 0.8% concentrations of *M. arvensis* essential oil respectively (Table 1). *M. arvensis* essential oil

showed significant (F = 209.88, P<0.01) repellency against *S. zeamais* adults.

3.2. Fumigant toxicity

Fumigation of *T. ammi* and *M. arvensis* essential oils caused toxicity by vapour action. Median lethal concentrations (LC_{50}) were 0.385 and 0.323 µl/cm^3 air for *T. ammi* essential oil after 24 and 48 h of exposure respectively (Table 2). Regression analysis showed concentration-dependent mortality in *S. zeamais* adults against *T. ammi* essential oil (F = 246.72 for 24 h and 269.33 for 48 h; P<0.01) (Table 3). Median lethal concentrations (LC_{50}) were 0.274 and 0.214 µl/cm^3 air for *M. arvensis* essential oil after 24 and 48 h of exposure respectively (Table 2). Regression analysis showed concentration-dependent mortality in *S. zeamais* adults against *M. arvensis* essential oil (F = 276.34 for 24 h and 294.53 for 48 h; P<0.01) (Table 3). The index of significancy of potency estimation, g-value indicates that the mean value is within the limits of all probabilities (P<0.1, 0.5 and 0.01) as it is less than 0.5. Values of t-ratio greater than 1.6 indicate that the regression is significant. Values of heterogeneity factor less than 1.0 denotes that model fits the data adequate.

Table 1. Repellency of *T. ammi* and *M. arvensis* essential oil against *S. zeamais* adults.

Oils	Concentration (%)	Percent Repellency (PR) * Mean±SD	Preference Index** (PI)
T. ammi	0.2	74.33±3.14	- 0.74
	0.4	98.33±2.03	- 0.98
	0.8	100±0.00	- 1.0
	1.6	100±0.0	- 1.0
M. arvensis	0.2	78.66±2.45	- 0.78
	0.4	94.83±0.89	- 0.94
	0.8	100±0.0	- 1.0
	1.6	100±0.0	1.0

*Percent repellency (PR) was calculated as: PR = (C-T)/(C+T) ×100; Where C = number of insects in the untreated halves and T = number of insect in treated halves; **Preference index (PI) was calculated as: PI = (percentage of insects in treated halves - percentage of insects in untreated halves)/ (percentage of insects in treated halves + percentage of insects in untreated halves). PI value between -1.0 to -0.1 indicates repellant essential oil, -0.1 to +0.1 neutral essential oil and +0.1 to +1.0 attractant essential oil.

Table 2. Fumigant and contact toxicity of *T. ammi* and *M. arvensis* essential oil against *S. zeamais* adults.

Oils	Toxicity	Exposure period (h)	LC_{50}[a]	g-value	Heterogeneity	t-ratio
T. ammi	Fumigant toxicity	24	0.385	0.17	0.33	3.08
		48	0.323	0.18	0.35	3.34
	Contact toxicity	24	0.317	0.17	0.30	3.64
		48	0.278	0.19	0.31	3.69
M. arvensis	Fumigant toxicity	24	0.274	0.19	0.33	4.03
		48	0.214	0.20	0.36	3.68
	Contact toxicity	24	0.206	0.20	0.31	3.46
		48	0.169	0.19	0.33	3.84

[a] µl/cm^3 for fumigant and µl/cm^2 for contact toxicity.

Table 3. Regression analysis of fumigant and contact toxicity of *T. ammi* and *M. arvensis* essential oil against *S. zeamais* adults.

Oil	Toxicity	Exposure period	Intercept	Slope	Regression Equation	Correlation coefficient
T. ammi	Fumigant toxicity	24 h	- 4.73	5.67	Y = - 4.73+5.67X	0.99
		48 h	2.17	4.88	Y = 2.17+4.88X	0.98
	Contact toxicity	24 h	- 4.99	5.13	Y = - 4.99+5.13X	0.99
		48 h	4.13	3.97	Y = 4.13+3.97X	0.99
M. arvensis	Fumigant toxicity	24 h	- 4.08	5.66	Y = - 4.08+5.66X	0.98
		48 h	5.33	7.18	Y = 5.33+7.18X	0.99
	Contact toxicity	24 h	4.89	4.40	Y = 4.89+4.40X	0.98
		48 h	6.68	6.11	Y = 6.68+6.11X	0.99

3.3. Contact toxicity

T. ammi and *M. arvensis* essential oils caused contact toxicity in *S. zeamais* adults. Median lethal concentration (LC_{50}) of *T. ammi* essential oil was 0.317 and 0.278 µl/cm^2 area against *S. zeamais* adults after 24 and 48 h of exposure respectively (Table 2). Regression analysis showed concentration-dependent mortality in *S. zeamais* adults against *T. ammi* essential oil (F = 267.64 for 24 h and 309.23 for 48 h; P<0.01) (Table 3). Median lethal concentration (LC_{50}) of *M. arvensis* essential oil was 0.206 and 0.169 µl/cm^2 area against *S. zeamais* adults after 24 and 48 h of exposure respectively (Table 2). Regression analysis showed concentration-dependent mortality in *S. zeamais* adults against *M. arvensis* essential oil (F = 244.14 for 24 h and 286.65 for 48 h; P<0.01) (Table 3). The index of significancy of potency estimation, g-value indicates that the mean value is within the limits of all probabilities (P<0.1, 0.5 and 0.01) as it is less than 0.5. Values of t-ratio greater than 1.6 indicate that the regression is significant. Values of heterogeneity factor less than 1.0 denotes that model fits the data adequate.

3.4. Oviposition inhibition

Fumigation of *S. zeamais* adults with *T. ammi* and *M. arvensis* essential oils significantly reduced oviposition potential. Reduction in oviposition was 79.42 and 39.59% of the control when *S. zeamais* adults were fumigated with 40% and 80% of 24-h LC_{50} of *T. ammi* essential oil respectively (F = 242.37, P<0.01) (Table 4) Similarly, reduction in oviposition was 52.31 and 17.56% of the control when *S. zeamais* adults were fumigated with 40% and 80% of 48-h LC_{50} of *T. ammi* essential oil respectively (F = 293.81, P<0.01) (Table 4).

Table 4. Oviposition inhibitory activities of *T. ammi* and *M. arvensis* essential oil in *S. zeamais*.

Oil	Concentration	No. of progeny emerged Mean±SD	F-value (2,15)	Concentration	No. of progeny emerged Mean±SD	F- value (2,15)
T. ammi	Control	87.57±5.98 (100%)	242.37	Control	87.57±5.98 (100%)	293.81
	40% of 24h-LC_{50}	69.53±3.76 (79.42)		40% of 48h-LC_{50}	45.81±3.77 (52.31)	
	80% of 24h-LC_{50}	34.66±2.99 (39.59)		80% of 48h-LC_{50}	15.38±1.66 (17.56)	
M. arvensis	Control	87.57±5.98 (100%)	213.84	Control	87.57±5.98 (100%)	312.66
	40% of 24h-LC_{50}	61.13±4.07 (69.83)		40% of 48h-LC_{50}	40.17±3.16 (45.87)	
	80% of 24h-LC_{50}	38.97±3.01 (44.51)		80% of 48h-LC_{50}	19.88±1.73 (22.70)	

Values in parentheses indicate per cent change with respect to control taken as 100%.

Reduction in oviposition was 69.83 and 44.51% of the control when *S. zeamais* adults were fumigated with 40% and 80% of 24-h LC_{50} of *M. arvensis* essential oil respectively ($F = 213.84$, $P<0.01$) (Table 4) Similarly, reduction in oviposition was 45.87 and 22.7% of the control when *S. zeamais* adults were fumigated with 40% and 80% of 48-h LC_{50} of *M. arvensis* essential oil respectively ($F = 312.66$, $P<0.01$) (Table 4).

4. DISCUSSION

Plant derived volatiles have received much attention in the scientific community as a tool in stored grain insect pest management programme [5-14]. Essential oils and its components also have been evaluated for their role in insect pest management programme. Linalool and linalyl acetate exhibited significant fumigant toxicity to rice weevils [30]. In present study, repellant, insecticidal and oviposition inhibition activities of *T. ammi* and *M. arvensis* essential oils in *S. zeamais* was studied. *T. ammi* and *M. arvensis* essential oils showed significant repellant activity against *S. zeamais* adults. Similarly, Chaubey has evaluated *Zingiber officinale* and *Piper cubeba* essential oils for its repellent against *T. castaneum*. *Z. officinale* and *P. cubeba* essential oils have repelled the adults of *T. castaneum* [31]. Essential oil of *A. sativum* repelled *T. castaneum* and *S. oryzae* adults at very low concentration [12, 14]. *T. ammi* and *M. arvensis* essential oils induced high mortality in *S. zeamais* adults when treated by fumigation or contact methods. Fumigant toxicity effect of essential oils from five species of *Eucalyptus viz. E. camaldulensis*, *E. grandis*, *E. viminalis*, *E. microtheca* and *E. sargentii* have been studied against *S. oryzae* adults [32]. Both *T. ammi* and *M. arvensis* essential oils reduced progeny production in *S. zeamais* which ultimately may be reduced damage caused by the insect. Similarly, essential oils from the rhizomes of *Z. officinale* and berries of *P. cubeba* have been evaluated for developmental inhibitory activities against *T. castaneum*. Fumigation with sublethal concentrations of these essential oils reduced the oviposition potential of the adults and inhibited development of larvae to pupae and pupae to adults [31]. Essential oil of *A. sativum* has evaluated for its oviposition inhibitory activities against *T. castaneum*. *A. sativum* reduces oviposition potential of the adults when treated by fumigant method and contact method both [12].

Although mode of actions of essential oils and its constituents on stored grain insect pests have not been established, yet, recent researches have demonstrated the interference of essential oils and its monoterpenes with acetylcholinesterase activity in *S. oryzae* and *T. castaneum* [13, 14]. Essential oils are lipophilic in nature and can be inhaled or ingested. The rapid action against insect pests is indicative of a neurotoxic mode of action and interference with the neuromodulator octopamine or GABA-gated chloride channels [33, 34]. Several essential oil and its components act on the octopaminergic system of insects. Octopamine is a neurotransmitter, neurohormone and circulating neurohormone-neuromodulator, and its disruption results in total breakdown of the nervous system [35]. Thus, the octopaminergic system of insects represents a target for insect control. Low molecular weight terpenoids are too lipophilic to be soluble in the haemolymph after crossing the cuticle and the proposed route of entry is tracheae [36]. Most insecticides bind to receptor proteins in the insect and interrupt normal neurotransmission leading to paralysis and death. Low molecular weight terpenoids with different structures may also bind to target sites on receptors that modulate nervous activity [35].

5. CONCLUSION

Use of essential oils as an alternative in insect pest management programmes is a sustainable alternative as they can be obtained from nature. Essential oils can be used as contact toxicity, fumigant toxicity, repellent, oviposition inhibitory and developmental inhibitory agents. These act on various levels in the insects so possibility of generating resistance is low. Thus, *T. ammi* and *M. arvensis* essential oils can be used as an alternative of synthetic insecticides in the stored-grain insect pest management.

TRANSPARENCY DECLARATION

Author has declared that no conflict of interests exists.

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Diversity and distribution of rhizospheric bacteria associated with Devil's cotton (*Abroma augusta* L.) along with alterations induced by the abiotic environment

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ABSTRACT

Recent research on the pharmacological aspects of Devil's cotton (*Abroma augusta* L.) plant has increased its impetus, notwithstanding its usage by the traditional practitioners, in face of its over-exploitation. We attempted a study of the association ecology of pedospheric bacteria associated with its rhizosphere. A total of 9 (nine) bacteria were isolated from rhizospheric soil collected from 8 natural locations of the Brahmaputra valley, distributed in 4 districts of Assam, India. The association between rhizospheric bacteria and fungal biota (including endo-mycorrhizae and non-mycorrhizal fungi) with abiotic soil properties can be exclusively advocated for a broader utilization as an ecological indicator for the conservation of target plant species as discussed in the present paper.

Keywords: Abiotic factors; Culture-dependent approach; Microbiome; Plant health; Rhizosphere.

1. INTRODUCTION

The rhizosphere, or the narrow zone of soil affected by the presence of plant roots [1], with respect to species richness and community size is considered one of the most diverse microbial habitats [2]. The bacterial community dominating rhizosphere by playing important roles in soil formation, biogeochemical cycling of carbon, nitrogen, phosphorus, and other elements [3], nutrient acquisition, protection against adverse biotic-abiotic factors, and in plant growth promotion through, for instance, the production of plant hormones [4, 5] affect plant health; apart from removal or degradation of toxic and/or recalcitrant organic contaminants [6, 7]. Although, researchers have displayed widespread interest in understanding the diversity and function of the rhizopsheric microbial communities, most studies regarding rhizosphere microbiome have centered on crop/ model plants. Consequently, the rhizosphere of wildly occurring plant species for the most part remains unknown [3].

Abroma augusta L. also known as the *Devils' cotton plant* (English) is a medicinally important plant included in the angiospermic family Malvaceae (previously under family Sterculiaceae), mainly used for the treatment of various types of disorder in the traditional systems of medicine [8-10]. The medicament utility coupled with its wild, limited distribution has led to overexploitation of this plant; which showcases the urgent need to conserve this plant species, both in *in situ* as well as *ex situ* conditions [9, 10]. In terms of vegetative morpho-taxonomy; it is evergreen, quick-, pubescent shrub or a small tree with spreading, velvety branches. It occurs naturally in tropical Asia, South and eastern Africa and Australia [11]; in both wild and cultivated areas, throughout the warm and moister parts of the Indian sub-continent ranging from Punjab and Uttar Pradesh eastwards to Arunachal Pradesh, Assam, Meghalaya and Tripura, ascending to 1,200 m, and southwards in peninsular India [12, 13]. Nonetheless, aside from studies of the arbuscular mycorrhizal [9] and non-mycorrhizal fungal communities [10] associated with this plant, little is known of the bacterial diversity of the other microbial communities present in its rhizosphere.

A World Health Organization (WHO) survey, depicts that 70-80% of the world population especially from developing countries rely on natural products of medicinal plants for their health care [14]. With regard to the origin of natural products, these are either produced by plants or their associated microbes, generally phyllospheric, rhizospheric or endophytic bacteria [15]. The aim of the present study was to analyze the bacterial diversity naturally present in the rhizosphere associated with wild *Abroma augusta* L.

2. MATERIALS AND METHODS

2.1. Rhizosphere soil sampling, processing and physico-chemical characteristics

Owing to the scattered distribution of *Abroma augusta* L. [9, 10]; sites from eight natural locations/provenances of Brahmaputra valley, *viz*.- Titabor, Borholla, Namrup, Nagamati, Kokilamukh, Kaziranga, Amsoi and Jagiroad varying in the anthropogenic interferences were used for the study. Rhizospheric soil was collected naturally occurring in these geographic locations and distributed in 4 districts *viz.* Jorhat, Dibrugarh, Golaghat and Nagaon of Assam state in India (latitude 24° 8′ to 24° 2′ N and longitude 89° 42′ to 96° 0′ E). The majority of rain fall (1800 mm to 3000 mm) in these regions occur during monsoon period i.e., March through May, the heaviest precipitation comes with the southwest monsoon, which arrives in June, stays through September, and often causes widespread and destructive flooding [16]. From each individual plant, rhizospheric soil samples (at least three samples) were taken by digging out a small amount of soil (500 g) close to plant roots up to the depth of 15-30cm and these samples were stored in sterilized polythene bags at 10ºC for further processing in the laboratory (maximum time between sampling and processing was 12 h) and physico-chemical analyses of soil.

The pH and soil temperature were measured for all soil samples using electronic digital pH meter (Eutech Instruments, Singapore) and soil thermometer (Jainco, India). Moisture content was determined by oven dry technique [17]. Organic carbon (%) estimation was done by Walkley-Black's method [18].

2.2. Isolation, identification and cultivation of bacteria

To ascertain the diversity of soil bacteria, qualitative analysis involving Warcup's soil plate method [19] and Waksman's soil dilution method [20] was used. Bacterial isolates were characterized using culture-dependent identification (morphology-based), Gram staining as well as biochemical reactions [21]. The isolates were observed using a microscope and were photo-micrographed using a camera.

2.3. Statistical analyses

All the data were analyzed statistically, Analysis of the diversity parameters with respect to Bacterial isolates/ species viz.-quantitative analysis such as density, frequency and abundance of rhizospheric soil myco-flora and diversity indices were computed based on standard methods and protocols [22, 23]. Pearson's coefficient of correlation was calculated to study the relationship

between different variables. For the statistical analyses, MS Excel 2007 was used.

3. RESULTS AND DISCUSSION

The physico-chemical parameters, viz. - *p*H, moisture content, soil temperature, electrical conductivity, humidity and organic carbon of all the locations where *A. augusta* was occured naturally have been discussed in our previous research work [9, 10] where the diversity and distribution of rhizospheric non-mycorrhizal myco-biota as well as arbuscular mycorrhizae associated with the target plant species was assessed.

Our present study revealed the presence of a total of 9 (nine) of 7 (seven) genera of bacteria (see Table 1; Plate 1), *viz*. - *Streptococcus* sp. 1, *Streptococcus* sp. 2, *Streptobacillus* sp., *Pseudomonas* sp., *Bacillus* sp. 1, *Bacillus* sp. 2, *Streptomyces* sp., *Serratia* sp. and *Micrococcus* sp. inhabiting the rhizopshere of *Abroma augusta* L. The morphological characteristics of bacterial isolates reported in the collected soil samples have been enumerated and discussed in Table 1. Habitat was observed to influence the occurrence of bacteria with maximum (8) occurrence of bacterial isolates at roadsides and minimum at foothills, fallows and riverine areas (1, each) (see Table 2). With respect to elevation (expressed in metres above sea level or m asl), maximum inhabitance of diverse bacteria were observed in the low elevation range, i.e. 50-80 m asl (23) with maximum (4) at 57 m asl with *Pseudomonas* sp. exhibiting maximum (8 elevation sites) variation in distribution (See Table 2, Figures 1-4).

Table 1. Morphological and biochemical characteristics of bacterial isolates in the collected soil samples of *Abroma augusta* L.

Sl. No.	Species	Colony morphology	Gram's reaction	Methyl red test	Catalase test	Oxidase test	Glucose fermentation test	Nitrate reduction test
1.	*Streptococcus* sp. 1	Flattened, depressed centre, entire, round, dull white	+	ca	-	ca	+	ca
2.	*Streptococcus* sp. 2	Flattened, depressed centre, wavy, lobate, white	+	ca	-	ca	+	ca
3.	*Streptobacillus* sp.	Pleomorphic, filamentous rod. Fusiform; develop characteristic lateral bulbar swellings	-	-	-	ca	+	ca
4.	*Pseudomonas* sp.	Round, translucent whitish, bright, button shaped colonies	-	-	+	+	-	+
5.	*Bacillus* sp. 1	Punctiform, irregular, opaque, whitish, raised	+	-	+	+	+	+
6.	*Bacillus* sp. 2	Punctiform, irregular, opaque, whitish, flat	+	-	+	-	+	+
7.	*Streptomyces* sp.	Clumpy, depressed, whitish colonies; Rods; form substrate and aerial mycelium	+	+	+	ca	ca	+
8.	*Serratia* sp.	Red coloured, round, irregular, elongated colonies	-	+	+	-	+	+
9.	*Micrococcus* sp.	White coloured, round, small	+	ca	+	+	-	+

N.B.: +: positive result, -: negative result, ca: could not be ascertained.

Table 2. Habitat - and elevation-wise natural occurrence and diversity of bacterial isolates in rhizosphere of *Abroma augusta* L.

Habitat							Elevation (m asl)															
Fallow land	**Foot hills**	**Forest fringe area**	**Paddy field**	**River side**	**Road side**	**Bacterial isolates**	**56**	**57**	**63**	**65**	**66**	**68**	**70**	**72**	**73**	**74**	**75**	**78**	**88**	**117**	**118**	**130**
					+	*Streptococcus* sp. 1		+		+						+						
+			+		+	*Streptococcus* sp. 2	+	+					+	+								
		+			+	*Streptobacillus* sp.					+	+	+		+							
		+	+		+	*Pseudomonas* sp.					+	+	+		+		+	+	+			
	+		+		+	*Bacillus* sp. 1											+		+	+	+	+
					+	*Bacillus* sp. 2		+		+						+						
				+		*Streptomyces* sp.			+													
					+	*Serratia* sp.		+														
					+	*Micrococcus* sp.				+												
1	1	2	3	1	8	Total	1	4	1	3	2	2	3	1	2	2	2	1	2	1	1	1

Table 3. Location wise natural occurrence, frequency and diversity indices of bacterial isolates in rhizosphere of *Abroma augusta* L.

Locations/ Sites	**Titabor**	**Borholla**	**Namrup**	**Nagamati**	**Kokilamukh**	**Kaziranga**	**Amsoi**	**Jagiroad**	**Frequency (%)**
Bacterial isolates									
Streptococcus sp. 1						+			6.25
Streptococcus sp. 2						+		+	18.75
Streptobacillus sp.							+		12.5
Pseudomonas sp.	+	+					+		18.75
Bacillus sp. 1		+	+	+					18.75
Bacillus sp. 2						+			6.25
Streptomyces sp.					+				6.25
Serratia sp.						+		+	6.25
Micrococcus sp.						+	+		6.25
Species richness (Unique)	0	2.5	1	1	0	3.4	3	1	
Diversity Index (Region wise)	0	0.02	0	0	0	0.03	0.07	0.02	

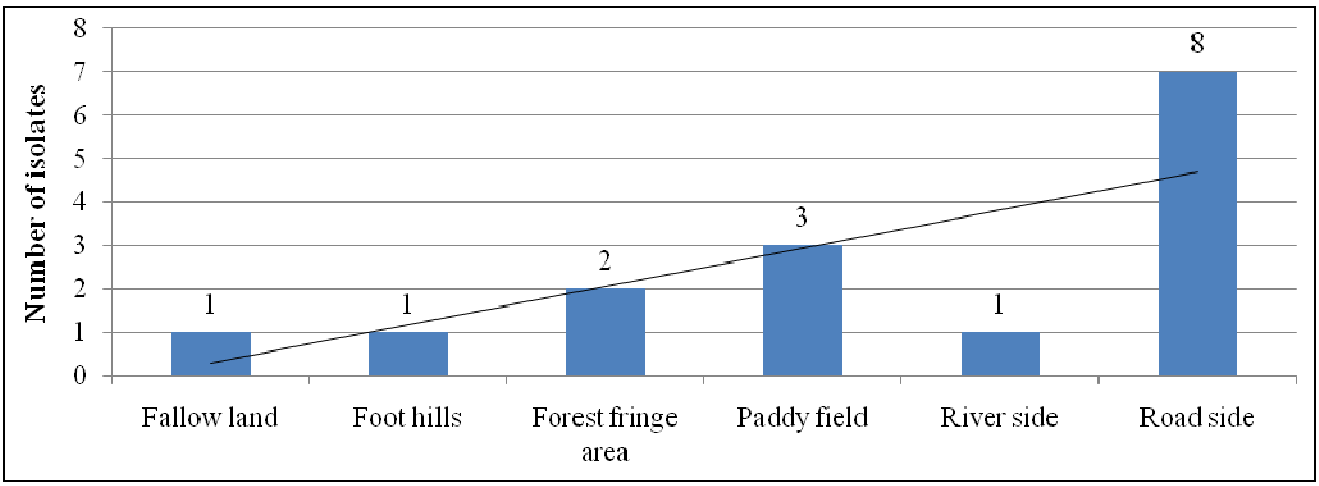

Figure 1. Habitat wise variation in bacterial isolates (on an average data).

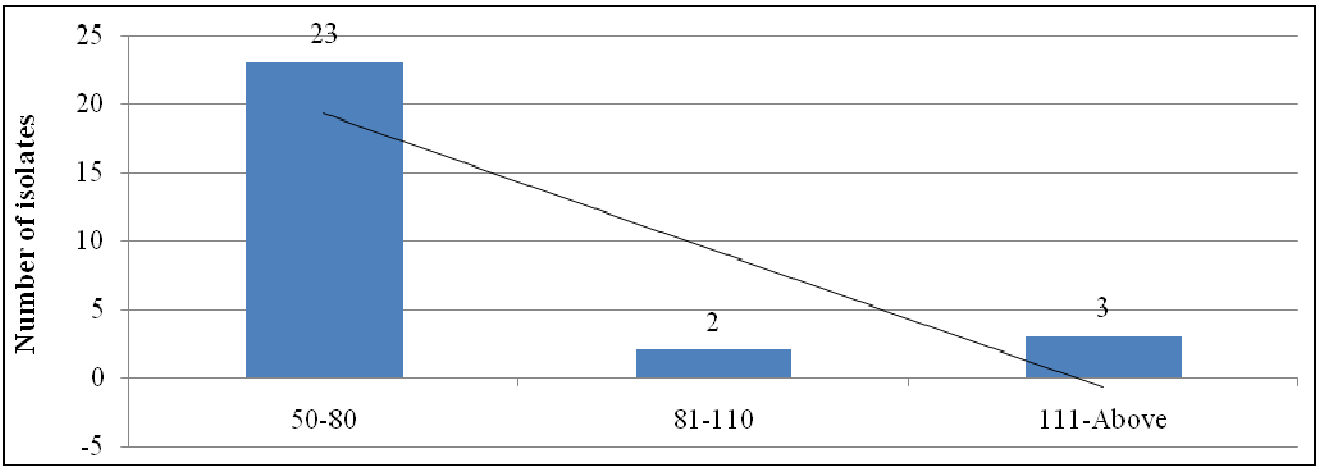

Figure 2. Elevation range wise variation in occurrence of bacterial isolates.

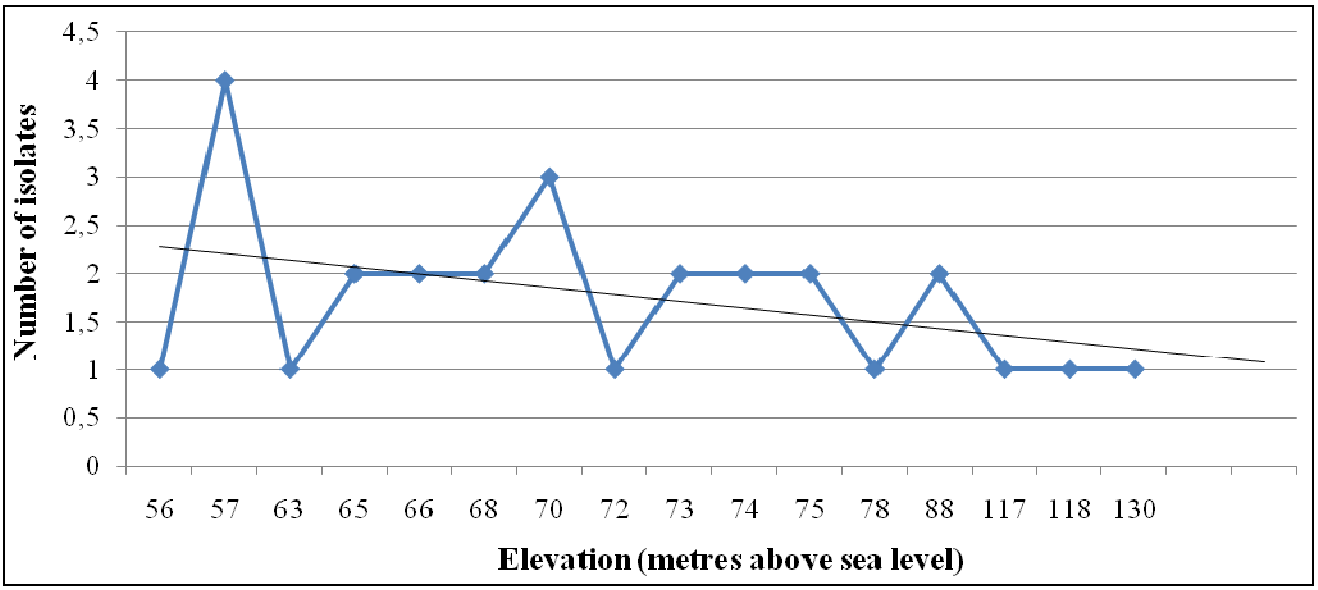

Figure 3. Elevation wise variation in bacterial isolates (on an average data).

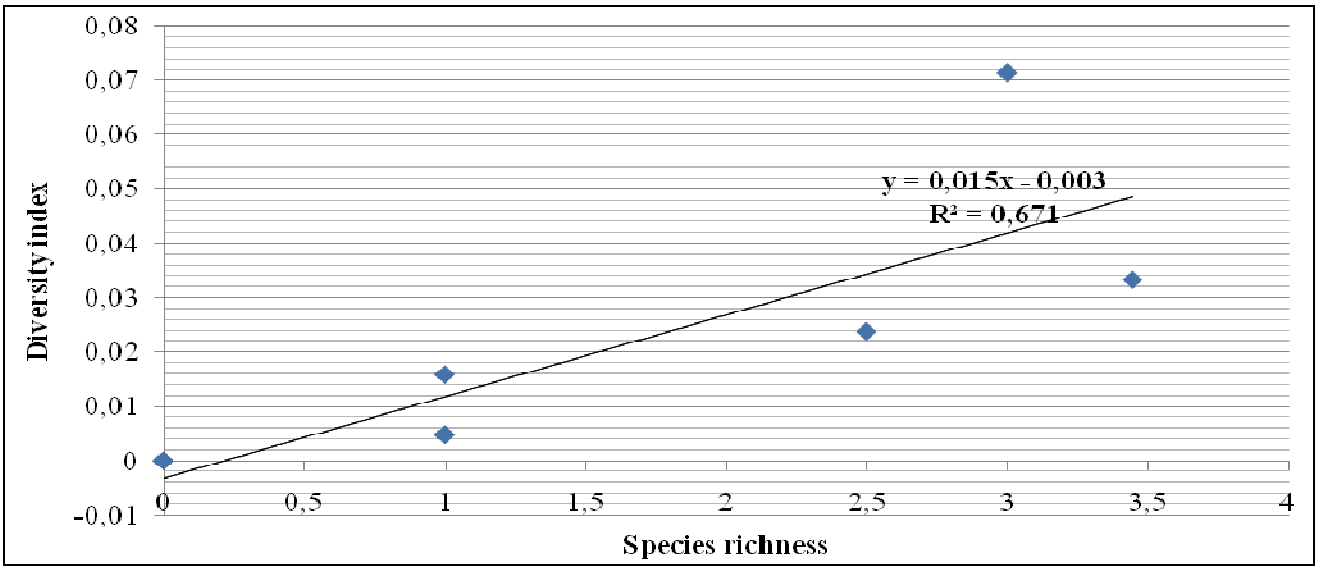

Figure 4. Scatter diagram - Species Richness *v/s* diversity Index.

The bacteria *Streptococcus* sp. 2, *Pseudomonas* sp. and *Bacillus* sp. 1 (3 isolates, each) were observed in larger numbers in contrast to *Streptococcus* sp. 1, *Bacillus* sp. 2, *Serratia* sp. and *Micrococcus* sp. (1 isolate, each). The natural occurrence (%) of bacterial isolates was maximum (18.75) in *Streptococcus* sp. 2, *Pseudomonas* sp. and *Bacillus* sp. 1; while it presented lower values (6.25) in *Streptococcus* sp.1, *Bacillus* sp. 2, *Streptomyces* sp., *Serratia* sp. and *Micrococcus* sp. the location Kaziranga exhibited greater diversity (5 genera) of bacteria, species richness (3.4) and diversity index (0.03); while Titabor and Kokilamukh had lower values (1,0,0 respectively) (see Table 3).

With regard to correlation studies between the abiotic factors (i.e. elevation, pH, electrical conductivity, temperature, organic carbon, humidity and moisture content) and biotic indices (Species richness and Diversity index); it was revealed that elevation and temperature had depressing effect on the bacterial communities, while electrical conductivity had constructive effect (see Table 4).

Table 4. Pearson's correlation matrix amongst various external environmental factors along with ecological indices of rhizospheric bacterial in study sites harboring *Abroma augusta* L.

	Elevation	pH	Electrical conductivity	Temperature	Organic Carbon	Humidity	Moisture content	Species richness	Diversity index
Elevation	1								
pH	-0.320	1							
Electrical conductivity	-0.716	0.091	1						
Temperature	0.071	-0.746	0.381	1					
Organic Carbon	0.039	0.181	-0.094	-0.583	1				
Humidity	-0.363	-0.299	0.720	0.712	-0.342	1			
Moisture content	-0.233	-0.255	0.643	0.562	-0.053	0.938	1		
Species richness	-0.138	-0.199	0.051	-0.058	0.206	0.180	0.296	1	
Diversity index	-0.426	0.129	0.127	-0.220	-0.057	-0.041	-0.059	0.775	1

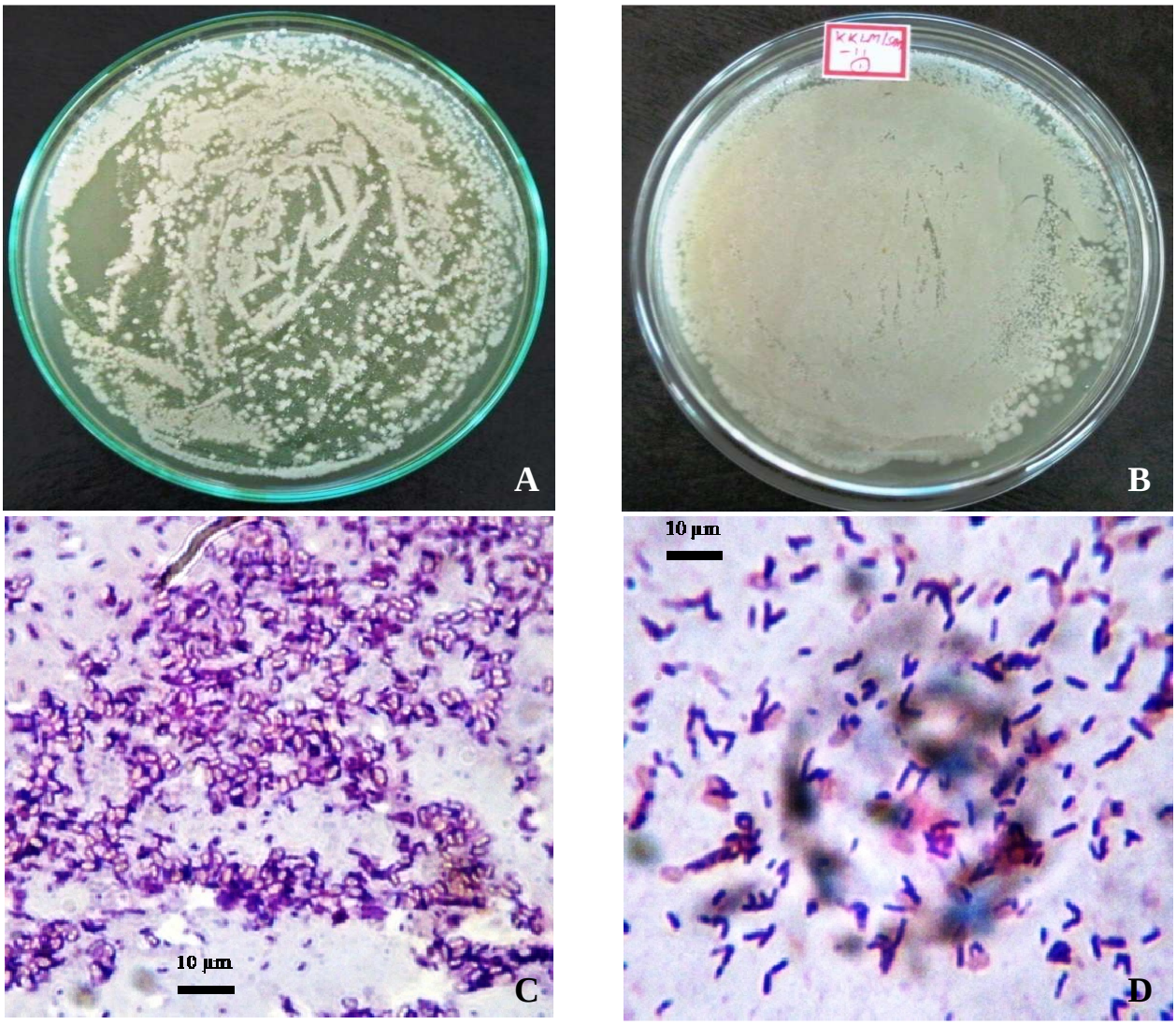

Plate 1. A: Colony of *Bacillus* sp., B: Colony of *Pseudomonas* sp., C: *Bacillus* sp., D: *Bacillus* sp.

In this work, the diversity within the rhizosphere bacterial community associated with the Devils' cotton plant species from the North-Eastern part of Indian sub-continent was explored using culture-dependent approaches. Since, the assembly of microbial communities in the rhizosphere can be affected by human activities such as the input of fertilizers and pesticides [3], sites varying in the anthropogenic interferences were also used for the study. In recent years, bioactive metabolites from medicinal plants have gained global attention. Bioactive metabolites are produced by medicinal plant or associated microbes. These bioactive metabolites are involved in symbiotic association with the host plant [24]. Bacteria produce bioactive metabolites exhibiting activities against phyto-pathogens as well as against bacteria, fungi, viruses, protozoans affecting humans and animals [15].

Out of the isolated, a *Pseudomonas* sp. (MSML/RFRI/Ps-1) was hypothesized as putative and multiplication was carried out through bacterial cultivation technique by using growth curve after specific time intervals of one hour [25]. The inoculum of bacteria was taken in stationery phase (10-11 hrs.) and has been tried for bio-inoculation experiments to assess their effect on accumulation of bioactive phyto-compunds, the data of which will be communicated in future by the authors.

4. CONCLUSIONS

This study confirmed the presence of certain bacteria in the rhizosphere of the target plant species; which can be further utilized for bio-inoculation studies; thereby, enhancing the conser-vation aspect of the plant. The association between

rhizospheric microbial biota including bacteria, endomycorrhizae and non-mycorrhizal fungi vis-à-vis abiotic soil properties can be exclusively advocated for a broader utilization as an ecological indicator for the conservation of target plant species.

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AUTHORS' CONTRIBUTION

VP has conceptualized the study and identified the bacterial isolates; VP and AJS have equally contributed in respect to survey, conduction of laboratory work and manuscript preparation. The final manuscript has been read and approved by both the authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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Research Article

Development of digital elevation model for Okomu National Park, Nigeria

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ABSTRACT

The type of soil, fauna and flora species that are found in an environment is affected by the elevation characteristics of the land. However, the ability to provide techniques and model that will effectively explain the elevation patterns of protected areas will aid sustainable management of the forest and its resources. This study developed Digital Elevation Model (DEM) for Okomu National Park, Nigeria. Point coordinates (2,272) with their respective elevations were randomly obtained covering the entire study area. Interpolated natural neighbour algorithm of the Quantum Geographic Information System was used to generate Digital Elevation Model for the National Park from the elevation data. Topographic map was extracted from the DEM at an interval of 10 m from one another. It was observed that the elevation in the study area ranged from 19 m to 105 m with an average of 56.32 m above sea level. Hence, the parkland is regarded as a gentle slope. This study revealed that the study area is not prone to flood or runoff due to its gentle slope nature. Therefore, this study is recommended as baseline information for ecological management as well as guide in the development of conservation strategies for flora and fauna species in the study area.

Keywords: Coordinates; Environment; Elevation; Forestry; Geographic information system; Global positioning system; Wildlife.

1. INTRODUCTION

Digital elevation models (DEMs) are computerized way of representing the Earths relief [1]. In a broader sense, DEMs can generally be described as spatially geo-referenced elevation data set that aids the encoding and representations of ground surface topography or terrain for environmental modeling purposes [2, 3]. They are also directly compatible with remotely sensed data sources; making it possible to represent intricate terrain units [2]. However, DEMs can also be used alongside other spatial data in geographic information systems (GIS) for advanced analysis [1].

In recent years, the used and application of DEM is on the increase as a tool and product. However, it provides applicable data in diverse areas such as; topographic and land cover studies, geomorphology, biology, hydrology, reservoir-management etc. [3-5]. Modeling relief has become essential in environmental research [4], especially with the increase in floods, droughts, soil erosion and other environmental effects of climate change. Digital elevation model aids better understanding and visualization of landscape and its relationships.

However, DEM provides avenue for quantification of several physical relief features such as water and erosion volumes. Thus, DEM also provides data for statistical and/or empirical modelling [1].

Furthermore, it is necessary to employ DEM as a managerial tool in the field of forestry, wildlife and ecotourism management as it will help in visualizing the fundamental relationship between altitudes and soil characteristics that enable the survival of flora and fauna on it. For instance; at higher altitude more acidic and humus soils exist in the montane or subalpine levels [6], while lower elevations exhibit fewer terrestrial species due to the thick layer of dead fallen leaves covering the forest floor in the tropical rain forest regions [7, 8]. Similarly, air temperature decreases with increase in elevation, thus influence variations in the length of a growing season at altitudes [7, 8]. Soil temperature and moisture, rate of evapotranspiration, humidity and precipitation are also related to altitude; optimum growth of a plant can be achieved by determining its suitable altitude range [6, 8, 9]. This also influences the type of fauna species that will be found in the environment. However, detail information on the relief and DEM, which helps in understanding land cover of the study area and for further spatial analysis [3] was lacking. Therefore, this study aims at developing DEM for Okomu National Park of Nigeria to providing baseline information and tool for ecological and sustainable forest management.

2. MATERIALS AND METHODS

2.1. Study area

The study was carried out in Okomu National Park formally known as Okomu Sanctuary is forest block within the 1,082 km^2 Okomu Forest Reserve located in Ovia South-west Local Government Area of Edo State, Nigeria. The park is 45 km west of Benin City with aland area of about 181 km^2 [10]. It lies between latitude 6°14'57.55" N and 6°24'55.64" N and longitudes 5°09'28.09" E and 5°20'15.51" E. The park contains the last remaining low rain forest ecosystem in southwestern Nigeria, with annual rainfall between 1524 and 2540 mm. Endowed with a complex assemblage of flora and fauna species [11]. The vegetation is semi-deciduous forest. The park has four ranges which are; Julius, Iguowan, Arakwan and Babui creeks [12].

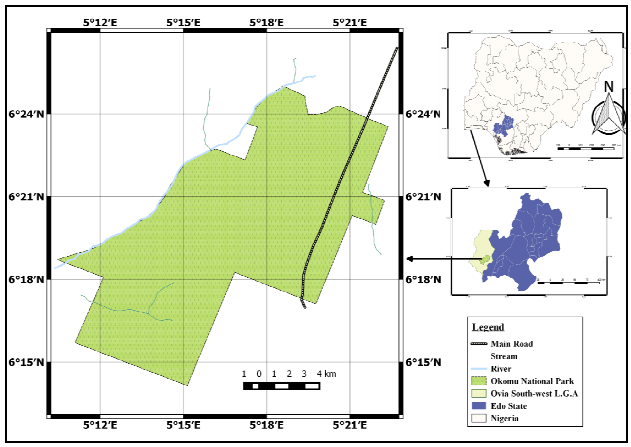

Figure 1. Map of Okomu National Park, Nigeria.

2.2. Data collection and analysis

Geographic Position System (GPS) was used to obtain point coordinates and elevations at various locations across the study area. A total of 2,272 points coordinates with respective elevation were obtained. The coordinates and elevations were saved in text (tab delimited) file format in Microsoft Excel spread sheet. Hence, was loaded into Quantum Geographic Information System (QGIS) for further analysis. The algorithm used includes interpolated natural neighbourof SAGA 2.1.2 geoalgorithm in QGIS to generate Digital Elevation Model for the National Park as was done by [3]. The Coordinate Reference System (CRS) was WGS84. The contour lines were extracted from DEM at aninterval of 10 m from one another.

3. RESULTS

The summary of statistics of the data collected in Okomu National Park was presented in Table 1. The coordinates ranged from latitude 6°14'57.55" N to 6°24'55.64" N and longitude 5°09'28.09" E to 5°20'15.51" E with elevation ranging from 19 to 105 m above sea level (asl). A total of 2,272 points coordinates with the corresponding elevation were obtained across various locations within the National Park. The GPS accuracy mean of 9 m and standard deviation of 3.961 was obtained for the data used for this

study. The graphical distribution (pie chart) of the elevation points used for development of the digital elevation model (DEM) was presented in Figure 2. The chart showed that 41% of elevation points (930) obtained from the study ranged from 30 m to 50 m asl. Elevation range of 50 to 70 m asl and 70 to 90 m asl accounted for about 24% and 23% respectively. Only about 4% (97) elevation points were less than 30 m asl. The mean elevation in is approximately 56.32 m asl.

Table 1. Summary of statistics of the spatial data for Okomu National Park.

Variables	Np	Mean (m)	SE	SD	Min	Max
Elevation (m)	2272	56.323	0.435	20.749	19	105
Accuracy (m)	2272	9	0.083	3.961	3	21
Latitude (N)	2272				6°14'57.55"	6°24'55.64"
Longitude (E)	2272				5°09'28.09"	5°20'15.51"

Where; Np = number of points, SE= Standard error, SD= Standard deviation, Root mean square, Min= minimum and Max = maximum, N= North, E = East. All coordinates are in degree minute second (DMS).

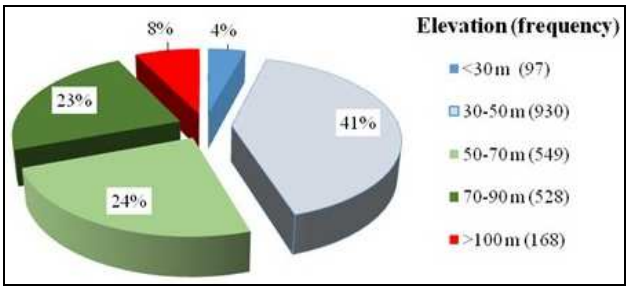

Figure 2. Distribution of elevation in Okomu National Park.

The digital elevation model and topographic map for the study area are showed in Figures 3 and 4 respectively. The colour graduated from blue (about 19 m asl) at the southern part of the national park to red (about 105 m asl) in the northern part. At the center, the colour interphase between red and blue with an elevation of about 60 m asl.

4. DISCUSSION

The descriptive statistics revealed that the average altitude of the park is about 56.32 m asl. This result is in disagreement with the work of Ejidike and Okosodo [11] that reported the average elevation of ONP was about 75 m. The elevation of Okomu National Park ranged from 19 m to 105 m (asl). This result was also incongruent with the reports of Aremu et al. [13], that the topography of ONP is gently ranging between 30 and 60 m asl and Akinsorotan et al. [14] who also reported the area to be within 300 meters asl. However, no research has been reported to obtain up to 2,272 points coordinates and elevation values in the study area. Hence, some parts of the National Park might not be effectively covered in the earlier research carried out by these researchers. Therefore, this study presents more reliable baseline information than earlier presented [11, 13, 14] because of the numbers of data used in this study.

Consequently, the effortwas directed towards obtaining Digital Elevation Model that will best describe the structure and/or nature of elevations of the sturdy area with a pattern of their distribution. Figure 2 reveals that only little portion on the park (8%) have elevation >90 m. This observation was confirmed by the DEM and topographic map (Figures 2 and 3, respectively), with only little portion around the northeastern part of the National park represented by the red colour.

The DEM revealed that greater percentage of the park land area has low altitude (between 30 to 90 m), this indicates that the forest land has gentle slope. This gentle slope helps in preventing runoff thereby retaining the soil organic matter and nutrient at the same time, prevent flooding since the land area is graduated from the south (30 m asl) to the north (100 m asl) (Figure 4). This result was in conformity with the report of Cobbina et al. [15] that assessed high conservation values in parts of Okomu's Extension I Concession and concluded that topographically, the Okomu landscape is

consistently flat and gently undulating throughout, with no steep slopes. Hence, the DEM displayed ONP land as one with less effect of soil erosion as a result of its plain landscape nature. This was also in agreement with work of Cobbina et al. [15], who affirmed that the risk of critical soil erosion in and around the Okomu National Park appears low as a result of the areas' low-lying topography.

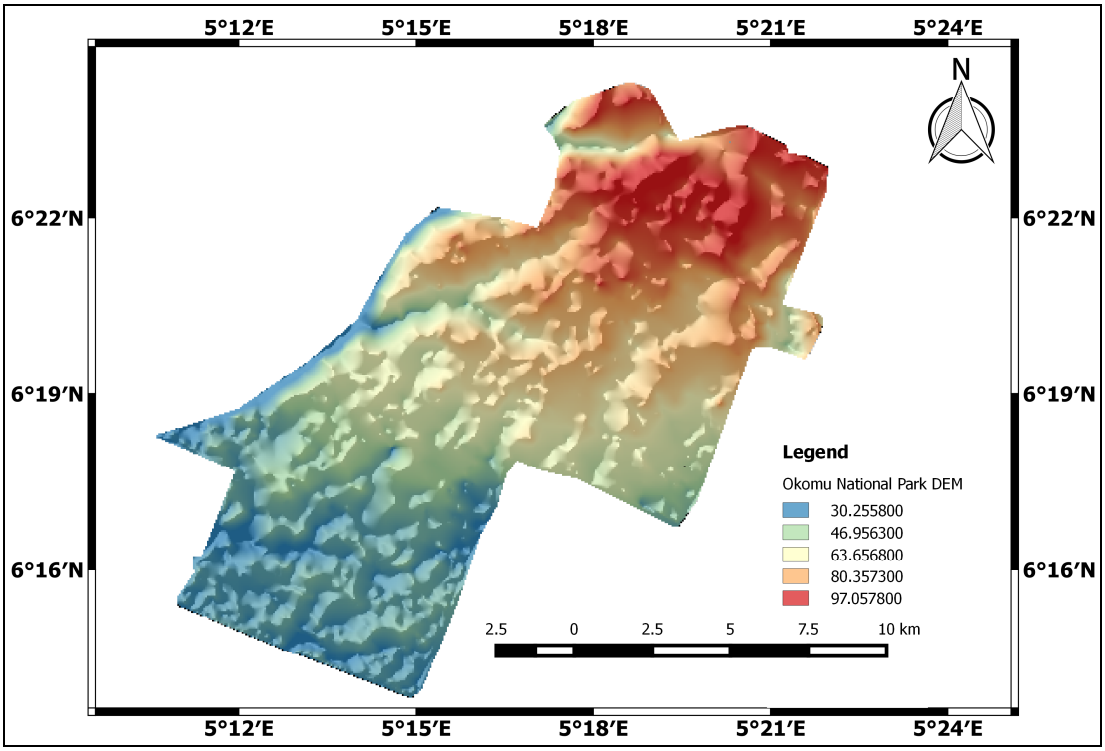

Figure 3. Digital Elevation Model of Okomu National Park.

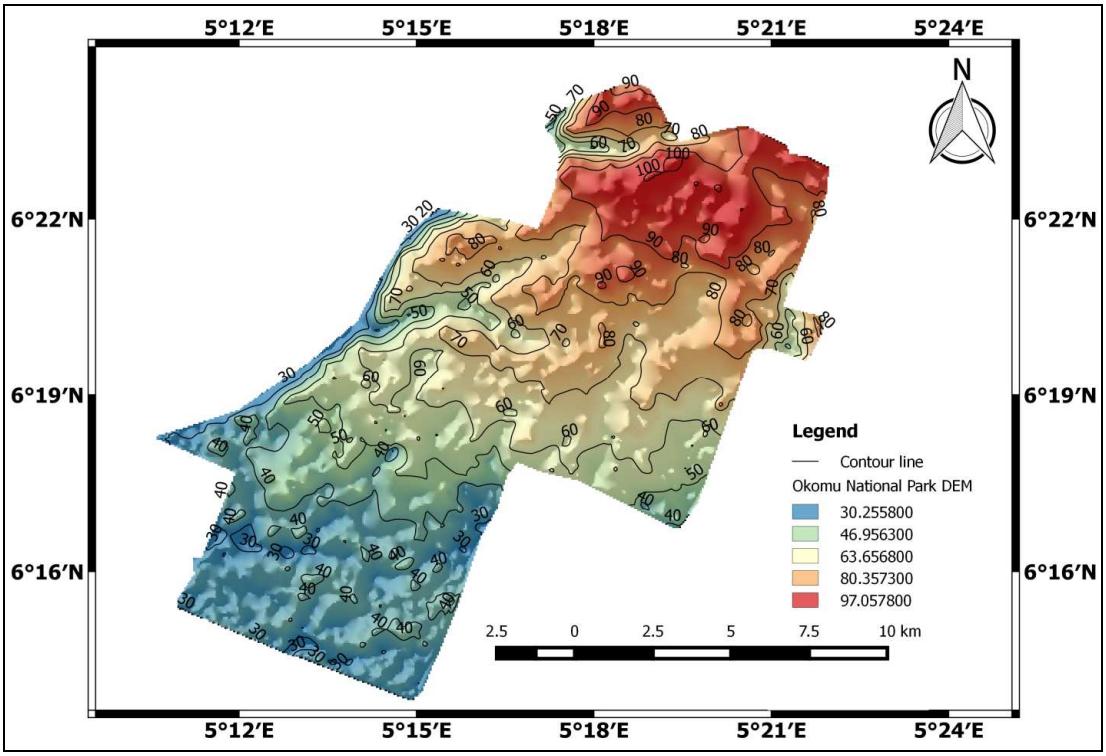

Figure 4. Contour lines of Okomu National Park on Digital Elevation Model.

Furthermore, altitudinal gradients change influences soil organic matter by controlling soil erosion, geologic deposition processes, soil water balance, species and biomass production of the native vegetation and cultivated plants [16]. However, Okomu National Park's high species richness and abundance [13], classification as an important bird area [17] and reference as habitat for numerous endangered flora and fauna species [18], might be attributed to high percentage of low altitudes in the National park. Similar result was reported by Heidari et al. [19], that evaluated herbaceous plant biodiversity in relation to physiographic factors (altitude, slope and aspect) in a protected area DalabIlam, they conclu-ded that, altitude have significant impact on the diversity and richness of herbaceous species. Karami et al. [20], Pourbabaei and Ahani [21] affirmed that diversity of plant species will decrease with increasing altitude. Additionally, the western part (border) of ONP is characterized with blue colour DEM (Figure 3) and low elevation contour (30 m asl) in Figure 4 due to the presence of Okomu river. The topographic map has towards the southwestern part of the national park has elevation ranged between 30 m asl to 40 m asl all through. This is suggesting that there is presence of river which could influence the altitude greatly. This has earlier been explained that River Okomu is responsible for this low altitude. The contour lines of the topographic map were extracted at interval of 10 m to accommodate differences in elevation of the study area unlike the topographic map of some large area of land produced by geographers which with larger interval. Extraction of ONP from such topographic map will always preclude detail of the study area. Therefore, the problem of over reliance on generalised for the development of topographic map for specific study and smaller the study area has been solved [3].

5. CONCLUSIONS

Digital Elevation Model was developed for Okomu National Park, Nigeria using geographic information system. The average elevation of the study area is 56.32 m asl. The digital elevation model could be used as a tool and/or baseline information for decision making as well as for design and implementation of habitat and ecotourism needs, conservation strategies and sustainable forest and ecological management.

This study has produced a detailed topographic map specifically for the study area, unlike generalized maps produced in most countries with no specific attention to National Parks and forest reserves.

This study therefore, provided baseline information on the relief of Okomu National Park, Nigeria. Hence, the park is not prone to flood or runoff because of the gentle nature of the slope in study area. Therefore, the DEM is recommended as tool for ecological management as well as guide in the development of conservation strategies for flora and fauna species in the study area. Similar study is recommended for other national parks.

AUTHORS' CONTRIBUTION

OC and JUE: data collection, interpretation of data, writing manuscript, material support and review of manuscript. OC and AAA: development of methodology, data analysis and development of DEM. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interests.

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Research Article

Flora of District Samba of Jammu and Kashmir State - I

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ABSTRACT

Samba is a newly formed district, located in Jammu and Kashmir State. The District was earlier a part of District Jammu of the State. Samba is situated on a range of Shivalik hills on the east of Jammu alongside the National Highway 1-A on the bank of river Basantar at a distance of forty kilometres from Jammu city. The present survey is undertaken since 2015 to explore the District taxonomically that will be helpful to carry out the research in other related fields. The current assessment of the taxonomic study of the area indicates that the District comprises of 677 species represented by 442 genera of 119 families of the flowering plants of which 77.1% are dicotyledons and 22.8% monocotyledons. The ten dominant genera of the District are *Cyperus* (18 spp.) followed by *Ipomoea* (13 spp.), *Ficus* (9 spp.), *Euphorbia* (8 spp.), *Fimbristylis* (8 spp.), *Acacia* (5 spp.), *Cassia* (5 spp.), *Lindernia* (5 spp.), *Polygonum* (5 spp.), *Medicago* (4 spp.). Poaceae (70 spp.) is the most dominant family followed by Fabaceae (63 spp.) and Asteraceae (52 spp.).

Keywords: Angiosperms; Floristcs; District Samba; Jammu and Kashmir; India.

1. INTRODUCTION

The founder of Samba was Malh Dev, the younger son of Rai Saidu of Lakhanpur who married into the family of Ghotar a local tribesman settled at Samba in 1400 A. D. But it remained under the supremacy of Jammu during the period of Hari Dev in 1816 A. D. Suchet Singh the younger brother of Raja Gulab Singh was made the Raja of Bandralta and Samba. Recently in 2006, Samba was formed a new district of Jammu and Kashmir state. Earlier this area was under district Jammu. District Samba (Fig. 1 and 2) is contiguous with tehsils Bishnah and Jammu of District Jammu on the west, Udhampur district on the north, Kathua district on the east and International border along the District with Punjab province of Pakistan on South.

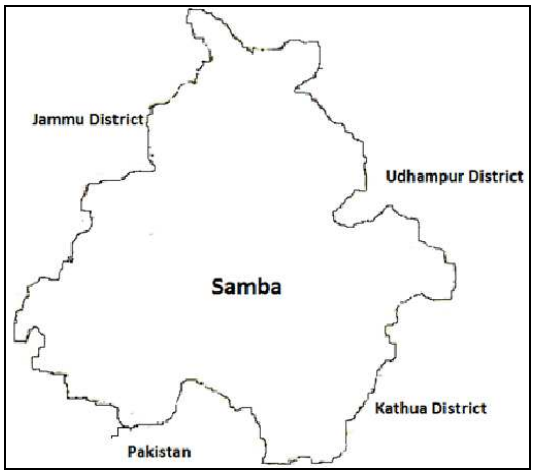

Figure 1. Map of district Samba of Jammu and Kashmir State, India.

The area of the District is 1002 sq. km and lies between 75° 11´ N longitude and 32° 56´ E latitude. The total population of the district is 3.1 lakh as per the census 2011. The area is mainly divided into two belts namely the outer plains on the south and the Shivaliks on the north. The national highway passes

through the middle of the district. Basanter and Devek are the sources of water particularly in rainy season. Samba is situated in the foothills of Shivaliks along the National Highway on the bank of river Basantar at the distance of 40 km from Jammu towards east.

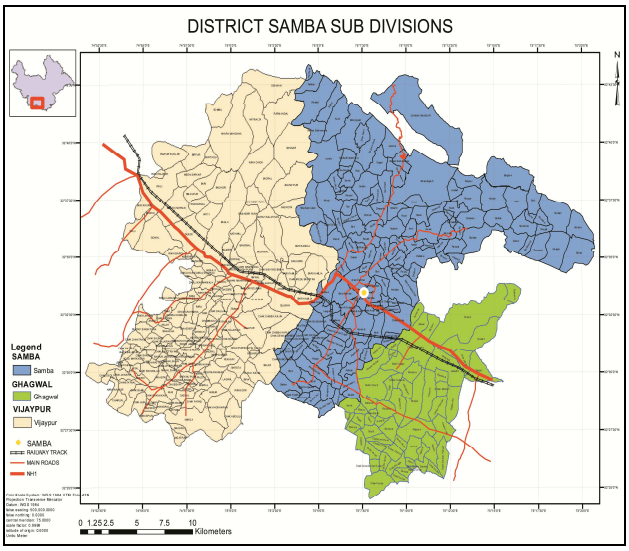

Figure 2. Map showing subdivisions of District Samba of Jammu and Kashmir, India.

1.1. Soil

Jammu and Kashmir have different types of soil. The soil of Samba district is typically like that of Jammu Shivaliks and Jammu plains because of its having some area like Shivaliks and other like that of Jammu plains. The soil is light to heavy with moderate fertility. Moisture is wanting in the northern part of Samba but the Southern part of Samba of Tehsil Ramgarh and its adjoining areas contain better moisture in the soil. The district is rich in limestone, quartzite, grit and clay [1].

1.2. Climate

The climate of district Samba is hot in summer and tolerably cold in winter with an exception of a very cold in the vicinity of high snow ranges on some of the northern part of the district. The excessive hot summer days are between May and June and rainy season prolongs upto middle of September. Most of the rainy days are in the month of July and August. The dry spell in the area follows from September. The climate of the area is based on precipitation, humidity and number of rainy days. This data is available in meteorological records [2-3].

1.3. Vegetation

The vegetation of district Samba is of subtropical type. The dominant species include *Buddleja asiatica*, *Carissa opaca*, *Dalbergia sissoo*, *Acacia nilotica*, *A. modesta*, *Justicia adhatoda*, *Lantana camara*, *Mallotus philippensis Murraya koenigii*, *Punica granatum*, *Woodfordia fruticosa Zizyphus mauritiana*, etc. The rare species are *Aegle marmelos*, *Aesculus indica*, *Bauhinia variegata*, *Bombax ceiba*, *Coriaria nepalensis*, *Crateva adensonii*, *Ficus bengalensis*, *F. religiosa*, *Butea monosperma*, *Mitragyna parvifolia*, *Phyllanthus emblica*, *Pyrus pashia*, *Rubus ellipticus*, *Terminalia chebula*, *Withania somnifera*, etc. Thorny bushes, evergreen shrubs, climbers and tall grasses. Most of the plants are broad leaved deciduous type. The district experience different types of vegetation due to variation in elevation, temperature and phytosociology of the species interrelationship since remote past. The most common species in the surroundings of Mansar lake are *Abrus precarorius*, *Abutilon indicum*, *Buddleja asiatica*, *Justicia adhatoda*, *Aesculus indica*, *Bauhinia variegata*, *Bombax ceiba*, *Cissampelos pariera*, *Dodonaea viscosa*, *Grewia aptiva*, *Murraya koenigii*, *Platanus orientalis*, *Telosma pallida*, *Toona ciliata*, *Nerium indicum*, *Ipomoea carnea*, *I. hederacea*, *I. purpurea*,

The characteristics herbaceous species of this area are *Aeginetia indica*, *Ageratum conyzoieds*, *Ajuga parviflora*, *Alternanthera pungens*, *Arabidopsis thaliana*, *Bidens bipinnata*, *Blumea laciniata*, *Centella asiatica*, *Convlovulus arvensis*, *Euphorbia hirta*, *E. prostrata*, *Galinsoga parviflora*, *Eclipta prostrata*, *Lactuca dissecta*, *Lindenbergia macrostachya*, *Malvestrum coromandelianum*, *Mazus delavayi*, *Medicago lupulina*, *M. polymorpha*, *Mentha longifolia*, *Murdannia nudiflora*, *Prunella vulgaris*, *Ranunculus arvensis*, *R. muricatus*, *Rubia cordata*, *Salvia plebeia*, *Saussurea heteromala*, *Scaligeria stewartiana*, *Veronica beccabunga*. The herbaceous flora in the surroundings of Samba city among other include *Achyranthes aspera*, *Anagallis arvensis*, *Dicliptera bupleuroides*, *Cannabis sativa*,

Cichorium intybus, Circium arvense, Galinsoga parviflora, Eclipta prostrata, Imperata cylindrica, Lactuca dissecta, Malva parviflora, Oxalis corniculata, Polygonum plebeium, Rubia cordata, Salvia plebeia, Saussurea heteromala, Silybum marianum, Solanum nigrum, S. surratense, Stellaria media. The roadsides species along the national highway generally found are *Ageratum conyzoides, Alternanthera pungens, Cannabis sativa, Coronopus didymus, Chenopodium alba, Datura innoxia, Dicanthium annulatum, Euphorbia hirta, Gomphrena celosoides, Polygonum hydropiper, Sonchus asper, S. oleraceous, Verbascum thapsus, Xanthium strumarium, Youngia japonica*.

Some weeds that grow on wastelands are *Achyranthes aspera, A. bidentata, Barleria cristata, Artemisa scoparia, B. prionitis, B. bipinnata, B. biternata, Calotropis procera, Clematis gouriana, Cleome viscosa, Coccinia indica, Commelina benghalensis, Dicliptera roxburghiana, Fumaria indica, Galium aparine, Geranium rotundifolium, Ipomoea cariaca, I. nil, I. pestigridis, Oxalis debilis, O. corniculata, Rorripa nasturtium-aquatcum, Paspalum scorbiculatum, Phyla nodiflora, Poa annua, Tephrosia purpurea, Trichodesma incucum, Verbascum thapsus, Withania somnifera.*

The common weeds that inhabit in rice fields within District Samba are *Ammania baccifera, A. senegalensis, Circium wallichii, Commelina benghalensis, Cyperus difformis, C. rotundus, C. sanguisorba, Eclipta prostrata, Echinochloa colonum, Lindernia ciliata, Ludwegia parviflora, Mollugo pentaphylla, Monochoria vaginalis, Nelumbo nucifera, Ranunculus muricatus, Sphenoclea zeylanica*.

The other species of weeds that are of common occurrence in wheat fields are *Anagallis arvensis, Fumaria indica, Lathyrus aphaca, L. sativa, Melilotus alba, M. indica, Medicago lupulina, M. polymorpha, Phalaris minor, Spergularia fallax, S. rubra, Stellaria media, Vicia faba, V. sativa, V. tetrasperma, Vaccaria pyramidata*. Some trees and shrubs inhabiting district Samba are *Azadirachta indica, Butea monosperma, Carissa opaca, Cordia dicotoma, Cravaeva adansonii, Dalbergia sissoo, Delonix regia, Grewia optiva, Lagerstroemia indica, Martynia annua, Mallotus philippensis, Melia azadirachata, Oroxylum indicum, Pongamia pinnata, Populus ciliata, Punica granatum, Putranjiva roxburghii, Ricinus communis, Rubus ellipticus, Terminalia bellirica, T. chebula Toona ciliata* [2, 4-5].

In past, plant explorers [6-18] have undertaken floristic studies in this area and some other parts of the Jammu and Kashmir State. Sharma and Kour [19] and Kour and Sharma [20], have studied some tree resources of this area. The area of the district samba was selected for present study at the district level as it has not so far been documented floristically. Samba district has a great diversity in its flora and some patches need a survey to update the floristic diversity of the State of Jammu and Kashmir.

2. MATERIAL AND METHODS

The area was visited several times for the collection of data during the years of 2015-2017. Over one hundred botanical tours were made in the district during different seasons. The identification of the species was made with the help of taxonomic literature available. Each species is provided with a citation and some important synonyms for providing more clarity on account of taxa under study. The species has been compared with type specimens of different Herbaria wherever necessary.

3. RESULTS

All the species collected from district Samba has been arranged in Bentham and Hooker's system of classification with a slight deviation whereever necessay. Each species is provided with a short citation, place of collection, elevation, flower colour and distribution.

Floristic Analysis

The number of species inhabiting in district Samba is 677 represented by 442 genera of 119 families of the flowering plants of which 522 are dicotyledons and 155 monocotyledons (Table I). The ten dominant families of the flowering plants are Poaceae, Fabaceae, Asteraceae, Cyperaceae, Euphorbiaceae, Lamiaceae, Scrophulariaceae, Convolvulaceae, Acanthaceae, Malvaceae and Moraceae (Table 2). The most dominant genus is *Cyperus* (18 spp.) followed by *Ipomoea* (13 spp.) and *Ficus* (9 spp.) (Table 3).

Table 1. Number of species, genera and families of angiosperms of flora of District Samba of Jammu and Kashmir State.

Group	Number of families	Number of genera	Number of species
Dicotyledons	99	344	522
Monocotyledons	20	98	155
Total	119	442	677

Table 2. Ten dominant families of flora of District Samba of Jammu and Kashmir State.

S. No	Name of the family	Number of genera	Number of species
1.	Poaceae	54	70
2.	Fabaceae	30	63
3.	Asteraceae	40	52
4.	Cyperaceae	7	37
5.	Euphorbiaceae	12	23
6.	Lamiaceae	18	22
7.	Scrophulariaceae	11	22
8.	Convolvulaceae	6	21
9.	Acanthaceae	12	17
10.	Malvaceae	7	12
11.	Moraceae	2	12
12.	Solanaceae	5	12

Table 3. Ten dominant genera of flora of Samba.

S. No.	Name of the genus	Number of species
1.	*Cyperus* L.	18
2.	*Ipomoea* L.	13
3.	*Ficus* L.	09
4.	*Euphorbia*	08
5.	*Acacia*	05
6.	*Cassia*	05
7.	*Lindernia*	04
8.	*Polygounum*	05
9.	*Medicago*	04
10.	*Vicia*	04

3.1. Enumeration of taxa

Ranunculaceae

Clematis gouriana Roxb.ex DC., Syst. Nat. 138. 1818; Hooker f., Fl. Brit. India 1: 4. 1872; Sharma & Kachroo, Fl. Jammu 1: 87. 1981.
Flower colour: Greenish-white
Flrs. & Frts.: August-October
Specimens examined: Mansar (350 m) Bhellum 25013
Distribution: India (Jammu & Kashmir), Ceylon, Java, Pakistan & Philippines

C. graveolens Lindl. in Journ. Hort. Soc. 1: 307. 1846; Hooker f., Fl. Brit. India 1: 4. 1872; Sharma & Kachroo, Fl. Jammu 1: 88. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 13. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 26. 2016.
Flower colour: Cream yellow
Flrs. & Frts.: June-October
Specimens examined: Mansar (350 m) Bhellum 25092
Distribution: India (Jammu & Kashmir), Temperate Himalaya, Pakistan

Ranunculus arvensis L., Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 20. 1872; Sharma & Kachroo, Fl. Jammu 1: 89. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 15. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 26. 2016.
Flower colour: Yellow
Flrs. & Frts.: March-July
Specimens examined: Bari Brahmana (300 m) Bhellum 25051
Distribution: India (Jammu & Kashmir), Asia & Europe

R. laetus Wall. ex Royle, Ill. Bot. Himal. Mount. 53. 1834. *R. laetus* Wall. ex Hooker f., Fl. Brit. India 1: 19. 1872; Sharma & Kachroo, Fl. Jammu 1: 89. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 15. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 27. 2016.
Flower colour: Bright yellow
Flrs. & Frts.: May-July
Specimens examined: Mansar (350 m) Bhellum 25071
Distribution: India (Jammu & Kashmir), Tibet & Afghanistan

R. muricata L., Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 20. 1872; Sharma & Kachroo, Fl. Jammu 1: 89. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda,

Kishtwar and Ramban Districts (Kashm. Himal.) 16. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 27. 2016.
Flower colour: Yellow
Flrs. & Frts.: March-May
Specimens examined: Vijaypur (300 m) Bhellum 25389
Distribution: India (Jammu & Kashmir), W. Asia, America & Europe

R. scleratus L., Sp. Pl. 551. 1753; Hooker f., Fl. Brit. India 1: 20. 1872; Sharma & Kachroo, Fl. Jammu 1: 89. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 16. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 28. 2016.
Flower colour: Yellow
Flrs. & Frts.: March-May
Specimens examined: Ramgarh (290 m) Bhellum 25034
Distribution: India (Jammu & Kashmir)

R. trichiphyllus Chaix in Vill., Hist. Pl. Daupf. 1: 335. 1786; Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 16. 1872; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 16. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 228. 2016.
Flower colour: White
Flrs. & Frts.: June-August
Specimens examined: Ramgarh (290 m) Bhellum 25097
Distribution: India (Jammu & Kashmir), Afghanistan, Baluchistan, Europe, N. Africa & Tibet

Menispermaceae

Cissampelos pareira L. var. *hirsuta* (Buch. Ham. ex Dc.) Forman in Kew Bull. 22: 356. 1968; Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 103. 1872; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 18. 2012.
Flower colour: Greemish yellow
Flrs. & Frts.: April-June & September-November
Specimens examined: Gura More (330 m) Bhellum 25298
Distribution: India (Jammu & Kashmir) & cosmopolitan in warm countries

Tinospora cordifolia (DC.) Miers in Ann. & Mag. Nat. Hist. Ser. 2, 7: 38. 1851. Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 97. 1872; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 18. 2012.
Flower colour: Greenish yellow
Flrs. & Frts.: July-September
Specimens examined: Rhaya (300 m) Bhellum 25425
Distribution: India (Jammu & Kashmir), Burma, Ceylon & Pakistan

Nymphaceae

Nymphaea alba L., Sp. Pl. 510. 1753: Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 114. 1872; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 20. 2012.
Flower colour: White
Flrs. & Frts.: June-September
Specimens examined: Ramgarh (290 m) Bhellum 25026
Distribution: India (Jammu & Kashmir)

N. nouchali Burm. f., Fl. Ind. 120. 1786; Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 114. 1872; Sharma & Kachroo, Fl. Jammu 1: 92. 1981.
Flower colour: White or white tinged red
Flrs. & Frts.: July-October
Specimens examined: Nandpur (290 m) Bhellum 25040
Distribution: India (Jammu & Kashmir), Africa, Hungary, Java & Philippines

Nelumbium nucifera Gaertn., Fruct. Sem. Pl. 1: 73. t. 19. f. 2. 1788; Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 116. 1872; Sharma & Kachroo, Fl. Jammu 1: 92. 1981.
Flower colour: Rose -pink
Flrs. & Frts.: August-September
Specimens examined: Ramgarh (290 m) Bhellum 25050
Distribution: India (Jammu & Kashmir), Australia, China, Japan, North Africa & Persia

Papaveraceae

Argemone maxicana L., Sp. Pl. 508. 1753: Sp. Pl. 555. 1753; Hooker f., Fl. Brit. India 1: 117. 1872; Sharma & Kachroo, Fl. Jammu 1: 92. 1981; Bhellum

et al., Fl. Exot. J & K 32. 2013.
Flower colour: Yellow
Flrs. & Frts.: April-May
Specimens examined: Samba (300 m) Bhellum 25100
Distribution: India (Jammu & Kashmir), America & other tropical countries

A. ochroleuca Sweet, Brit. Gard. 3: t. 242. 1829; Bhellum et al., Fl. Exot. J & K 33. 2013.
Flower colour: White or cream coulured
Flrs. & Frts.: April-June
Specimens examined: Mansar Lake (400 m) Bhellum 25116
Distribution: India (Jammu & Kashmir) & Mexico and introduced in India

Fumariaceae

Fumaria indica (Haussk.)Pugsley in J. Linn. Soc. 44: 313. 1919; Sharma & Kachrroo, Fl. Jammu 1: 93. 1981; Bhellum et al., Fl. Exot. J & K 44. 2013; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 23. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 30. 2016.
Flower colour: Pinish purple
Flrs. & Frts.: March-May
Specimens examined: Ramgarh (290 m) Bhellum 25125
Distribution: India (Jammu & Kashmir), Afghanistan, Baluchistan, Mangolia, Persia & Turkestan

Brassicaeae

Arabidopsis thaliana (L.) Heyne in Holl. & Heynh., Fl. Sachs 1: 538. 1842; Sharma & Kachrroo, Fl. Jammu 1: 96. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 24. 2012.
Flower colour: White
Flrs. & Frts.: March-April
Specimens examined: Samba (300 m) Bhellum 25002
Distribution: India (Jammu & Kashmir), Afghanistan, Australia, S. Africa, & W. Asia

Capsella bursa-pastoris Medik., Pflanzengatt. 1: 85. 1792.; Hooker f., Fl. Brit. India 1: 159. 1872; Sharma & Kachrroo, Fl. Jammu 1: 96. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 26. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 33. 2016.
Flower colour: White
Flrs. & Frts.: Mansar (300 m) Bhellum 25035
Specimens examined: Samba (300 m) Bhellum
Distribution: India (Jammu & Kashmir), Asia, Europe & Persia

Cardamine flexuosa With., Bot. Arr. Brit. Pl. ed. 3, 3: 578. t. 3. 1796; Sharma & Kachrroo, Fl. Jammu 1: 95. 1981; Bhellum et al., Fl. Exot. J & K 55. 2013; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 34. 2016.
Flower colour: White
Flrs. & Frts.: April-June
Specimens examined: Vijaypur (300 m) 25080
Distribution: India (Jammu & Kashmir), Afghanistan, Isles of Europe, N. America, Pakistan & Asia

Coronopis didymus (L.) Sm., Brit. 2: 691. 1804. Sharma & Kachroo, Fl. Jammu 1: 95. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 27. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 35. 2016.
Flower colour: Pale green
Flrs. & Frts.: March-May
Specimens examined: Rahya Suchani (300 m) Bhellum 25091
Distribution: India (Jammu & Kashmir) & S. America

Descurainea Sophia (L.) Webb. et. Berth. in Engler & Prantl, Pflanzenf. 3, 2: 192. 1891; Sharma & Kachrroo, Fl. Jammu 1: 96. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 27. 2012; Bhellum et al., Fl. Exot. J & K 59. 2013; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 35. 2016.
Flower colour: Yellow
Flrs. & Frts.: March-April
Specimens examined: Mansar (300 m) Bhellum 25200
Distribution: India (Jammu & Kashmir), Africa, China, Europe, N & S. America & Central Asia

Eruca sativa Mill., Gard. Dict. ed. 8, 1: 1768; Hooker f., & T. Anders. in Hooker f., Fl. Brit. India 1: 158. 1872.

Flower colour: Pale yellow or Dull yellow
Flrs. & Frts.: March-April
Specimens examined: Samba (300 m) Bhellum 25095
Distribution: India (Jammu & Kashmir), Europe, North Africa, South America & Pakistan

Lepidium sativum L., Sp. Pl. 644. 1753; Hooker f., & T. Anders. in Hooker f., Fl. Brit. India 1: 159. 1872; Sharma & Kachrroo, Fl. Jammu 1: 97. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 28. 2012.
Flower colour: Light pink
Flrs. & Frts.: March-April
Specimens examined: Ramgarh (290 m) Bhellum 25220
Distribution: India (Jammu & Kashmir), Afghanistan, Pakistan & Sri Lanka

Rorippa indica (L) Hiern., Cat. Afr. Pl. Welw. 1: 26. 1886; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 28. 2012.
Flower colour: Yellow
Flrs. & Frts.: April-July
Specimens examined: Gaghwal (330) Bhellum 25241
Distribution: India (Jammu & Kashmir), Archipelago, China, Japan, Malayan & Philippines

R. palustris (L.) Besser, Enum. Pl. Volhyn. 1822. *Sisymbrium amphibium* L. var. *palustre* L., Sp. Pl. 657. 1753. *Rorippa islandica* auct. non Oeder; Borbas, Balaton Tav. 2: 392. 1900.
Flower colour: Yellow
Flrs. & Frts.: March-April
Specimens examined: Samba (300 m) Bhellum 25134
Distribution: India (Jammu & Kashmir),

R. nasturtium-aquaticum (L.) Hayek. Sched. Fl. Stir. Exs. 22. 1905; Meikle in Kew Bull. 97. 1954; Bennet, Name Changes Fl. Pl. 488. 1987; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 29. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 38. 2016. *Nasturtium officinale* R. Br. in Aiton, Hort. Kew. ed. 2, 4: 110. 1812; Hooker f., & T. Anders. in Hooker f., Fl. Brit. India 1: 161. 1872; Sharma & Kachrroo, Fl. Jammu 1: 93. 1981.
Flower colour: White
Flrs. & Frts.: May-August
Specimens examined: Rahya Suchani (Bhellum) 25261
Distribution: India (Jammu & Kashmir) & Temperate Europe

Sisymbrium iria L., Sp. Pl. 659. 1753; Hooker f., & T. Anders. in Hooker f., Fl. Brit. India 1: 150. 1872; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 29. 2012.
Flower colour: Yellow
Flrs. & Frts.: March-April
Specimens examined: Bari Brahmna (300 m) Bhellum 25295
Distribution: India (Jammu & Kashmir, Delhi, Rajastan), Afghanistan, Arabia, Europe & Mediterranean region

Capparidaceae

Capparis sepiaria L., Syst. 10(2): 1071. 1759; Hooker f., Fl. Brit. India 1: 177. 1872; Sharma & Kachroo, Fl. Jammu 1: 99. 1881.
Flower colour: pale yellow
Flrs. & Frts.: April-May
Specimens examined: Mansar (350 m) Bhellum 25299
Distribution: India (Jammu & Kashmir), Burma & Malay

Crataeva adansonii DC. subsp. odora (Buch.-Ham.) Jacobs. in Blumea 12: 198. 1964. *C. religiosa* Forst. f. var. *roxburghii* (R. Br.) Hooker f., & T. Anders. in Hooker f., Fl. Brit. India 1: 172. 1872.
Flower colour: Pale yellow
Flrs. & Frts.: April-May
Specimens examined: Samba (300 m) Bhellum 25301
Distribution: India (Jammu & Kashmir), Archipelago, China, Formosa, Malaya, Polynesia & Tropical Africa

Cleomaceae

Cleome brachycarpa Vahl. ex DC., Prodr. 1: 240. 1824; Sharma & Kachroo, Fl. Jammu 1: 98. 1981.
Flower colour: Yellow
Flrs. & Frts.: April-May
Specimens examined: Samba (300 m) Bhellum 25259
Distribution: India (Jammu & Kashmir), Arabia, North S. Africa & Pakistan

C. gynandra L., Sp. Pl. 671. 1753; Stewart, Ann. Cata. Vasc. Pl. W. Pak. & Kashm. 295. 1972; Sharma & Kachroo, Fl. Jammu 1: 98. 1981; Bhellum, Fl. ASC. Sgr. (Kashm.) India 40. 2016.
Flower colour: Yellow
Flrs. & Frts.: April-June
Specimens examined: Barhi Brahmna (300 m) Bhellum 25271
Distribution: India (Jammu & Kashmir), all Tropical Countries, Australia & South Africa

C. viscosa L., Sp. Pl. 672. 1753; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 31. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 40. 2016.
Flower colour: Yellow
Flrs. & Frts.: March-May
Specimens examined: Samba (300 m) Bhellum 25242
Distribution: India (Jammu & Kashmir), all Tropical Countries, China, Malaysia & Pakistan

Violaceae

Viola canescens Wall. ex Roxb., Fl. Ind. 2: 450. 1824; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 32. 2012.
Flower colour: pale Violet
Flrs. & Frts.: March-April
Specimens examined: Mansar (300 m) Bhellum 25104
Distribution: India (Jammu & Kashmir), Asia, Africa, China, Europe, Java & Pakistan

Polygalaceae

Polygala abyssinica R. Br. ex Freen in Mus. Senk. 2: 273. 1837; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 33. 2012.
Flower colour: Purple
Flrs. & Frts.: April-October
Specimens examined: Mansar (400 m) Bhellum 25149
Distribution: India (Jammu & Kashmir), Tropical and Subtropical Himalaya, Afghanistan, Abissinia to Natan

Caryophyllaceae

Arenaria serpyllifolia L., Sp. Pl. 423. 1753; Sharma & Kachrroo, Fl. Jammu 1: 105. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 34. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 42. 2016.
Flower colour: White
Flrs. & Frts.: Feburary-April
Specimens examined: Ramgarh (290 m) Bhellum 25156
Distribution: India (Jammu & Kashmir), Afghanistan, Asia, Europe, Nepal & W. Tibet

Cerastium glomeratum Thuill., Fl. Enu. Par. ed. 2, 226. 1799; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 35. 2012.
Flower colour: White
Flrs. & Frts.: May-July
Specimens examined: Samba (300 m) Bhellum 25303
Distribution: India (Jammu & Kashmir)

Polycarpaea corymbosa (L.) Lamk., Tab. Encycl. 2: 129. 1797; Sharma & Kachrroo, Fl. Jammu 1: 106. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 37. 2012.
Flower colour: White
Flrs. & Frts.: March-May
Specimens examined: Ramgarh (290 m) Bhellum 25402
Distribution: India (Jammu & Kashmir), America, Africa, Australia & Tropical Asia

Sagina apetala Ard. Animadv. Bot. Sp. 2: 22. t. 8. f. 1. 1763; Sharma & Kachrroo, Fl. Jammu 1: 105. 1981; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 43. 2016.
Flower colour: Green
Flrs. & Frts.: March-May
Specimens examined: Ramgarh (290 m) Bhellum 25025
Distribution: India (Jammu & Kashmir), Afghanistan, Europe, Pakistan & Turkey

Silene conoidea L., Sp. Pl. 418. 1753; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 37. 2012.

Flower colour: Pink
Flrs. & Frts.: March-April
Specimens examined: Ramgarh (290 m) Bhellum 25265
Distribution: India (Jammu & Kashmir), Nepal & Pakistan

Spergularia fallax Lowe, in Hook., Journ. Bot. 8: 289. 1886; Nair, Bull. Bot. Surv. India 9(1-4): 277. 1967; Sharma & Kachroo, Fl. Jammu 1: 105. 1981. *Spergula fallax* (Lowe) Krause in Sturm, Fl. Deutsch. (ed. 2) 5: 19. 1901.
Flower colour: White
Flrs. & Frts.: February
Specimens examined: Ramgarh (300 m) Bhellum 25275
Distribution: India (Jammu & Kashmir), Afghanistan, Africa, Arabia, Baluchistan, Egypt, Palesine & Persia

S. rubra (L.) J. & C. Presl. Fl. Cech. 94. 1819; Sharma & Kachroo, Fl. Jammu 1: 106. 1981. *Arenaria rubra* L., Sp. Pl. 423. 1753. *Spergula rubra* Edgew. & Hooker f. in Hooker f., Fl. Brit. India 1: 244. 1874.
Flower colour: Pinkish-violet
Flrs. & Frts.: February-April
Specimens examined: Ramgarh (300 m) Bhellum 25294
Distribution: India (Jammu & Kashmir), N & W Asia & Europe

Stellaria media (L.) Vill., Hist. Pl. Dauph. 3: 615. 1789; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 38. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 43. 2016.
Flower colour: White
Flrs. & Frts.: February-April
Specimens examined: Mansar (400 m) Bhellum 25332
Distribution: India (Jammu & Kashmir), Europe, Pakistan & W. Tibet

Vaccaria pyramidata (L.) Medik., Phil. Bot. 1: 96. 1789; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 39. 2012.
Flower colour: Pink
Flrs. & Frts.: March-April
Specimens examined: Ramgarh (290 m) Bhellum 25405
Distribution: India (Jammu & Kashmir), America, Asia minor, Europe, Pakistan & Siberia

Portulacaceae

Portulaca grandiflora Hook., in Bot. Mag. n.s. 3. 2885. 1829. Stewart, Ann. Cata. Vasc. Pl. W. Pak. & Kashm. 237. 1972.
Flower colour: Purple
Flrs. & Frts.: April-August
Specimens examined: Samba (300 m) Bhellum 25570
Distribution: India (Jammu & Kashmir, Punjab, U. P., Delhi, Maharastra) & Pakistan

P. oleracea L., Sp. Pl. 445. 1753; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 39. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 44. 2016.
Flower colour: Yellow
Flrs. & Frts.: April-August
Specimens examined: Samba (300 m) Bhellum 25102
Distribution: India (Jammu & Kashmir), North America and throughout the warm countries of the world

P. quadrifida L., Mant. Pl. 73. 1767; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 39. 2012.
Flower colour: Yellow
Flrs. & Frts.: May-September
Specimens examined: Samba (300 m) Bhellum 25105
Distribution: India (Jammu & Kashmir), Africa & Tropical Asia

Tamaricaceae

Tamarix dioica Roxb.ex Roth, Nov. Pl. Sp. 185. 1821 et Fl. Ind. 2: 101. 1832; Dyer in Hooker f., Fl. Brit. India 1: 249. 1872. Sharma & Kachroo, Fl. Jammu 1: 108. 1981.
Flower colour: Red
Flrs. & Frts.: April-May
Specimens examined: Ramgarh (290 m) Bhellum 25525
Distribution: India (Jammu & Kashmir), Burma & Pakistan

Elatinaceae

Bergia ammanioides Heyne ex Roth, Nov. Pl. Sp. Prassert. Ind. Or. ex Coll.Heynei 219. 1821. Hooker f, Fl. Brit. India 1: 251. 1872.
Flower colour: Red
Flrs. & Frts.: February-March
Specimens examined: Ramgarh (290 m) Bhellum 25179
Distribution: India (Jammu & Kashmir)

Malvaceae

Abutilon indicum (L.) Sweet, Hort. Brit. 54. 1826; Masters in Hooker f., Fl. Brit. India 1: 326. 1874; Sharma & Kachroo, Fl. Jammu 1: 111. 1981.
Flower colour: Yellow
Flrs. & Frts.: May-August
Specimens examined: Vijaypur (300 m) Bhellum 25343
Distribution: India (Jammu & Kashmir), Africa & Australia

A. ramosum Guill. in Perr. et A Rich., Fl. Senegamb. 1: 68. 1830; Masters in Hooker f., Fl. Brit. India 1: 328. 1874.
Flower colour: Yellow
Flrs. & Frts.: August-October
Specimens examined: Vijaypur (300 m) Bhellum 25397
Distribution: India (Jammu & Kashmir), Pakistan, Tropical Africa

Hibiscus hirtus L., Sp. Pl. 694. 1753; Masters in Hooker f., Fl. Brit. India 1: 326. 1874; Paul in Sharma et al., Fl. India 3: 329. 1993.
Flower colour: Rose-red
Flrs. & Frts.: July-September
Specimens examined: Vijaypur (300 m) Bhellum 25530
Distribution: India (Jammu & Kashmir), Malesia

H. lobatus (Murr.) O. Ktze., (Murray) Kuntze, Rev. Gen. Pl. 3, 2: 19. 1898; Sharma & Kachroo, Fl. Jammu 1: 112. 1981. *Solandra lobata* Murr. Comm. Soc. Reg. Sc. Goetting 6: 20. t. l. 1785.
Flower colour: White
Flrs. & Frts.: August-October
Specimens examined: Vijaypur (300 m) Bhellum 25572
Distribution: India (Jammu & Kashmir), Central Asia and Trpical Africa

H. vitifolia L. Sp. Pl. 696. 1753; Sharma & Kachroo, Fl. Jammu 1: 112. 1981.
Flower colour: Yellow with purple spotted at base
Flrs. & Frts.: September-October
Specimens examined: Vijaypur (300 m) Bhellum 25306
Distribution: India (Jammu & Kashmir)

Kydia calycina Roxb., Pl. Corom. 3: 11. t. 215. 1819 et Fl. Ind. 3: 188. 1832; Masters in Hooker f., Fl. Brit. India 1: 348. 1874; Sharma & Kachroo, Fl. Jammu 1: 110. 1981.
Flower colour: White
Flrs. & Frts.: July-October
Specimens examined: Samba (300 m) Bhellum 25600
Distribution: India (Jammu & Kashmir, Assam, Punjab, U. P. W. Panninsula), Burma

Malva parviflora L., in Boejer, Demonstr. Pl. Hort. Ups. 18. 1753; Masters in Hooker f., Fl. Brit. India 1: 321. 1874; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 41. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 47. 2016.
Flower colour: White or light pink
Flrs. & Frts.: February
Specimens examined: Ramgarh (300 m) Bhellum 25258
Distribution: India (Jammu & Kashmir), West Asia, Europe, North Africa

Malvastrum coromandelianum (L.) Garcke in Bonplandia 5: 295. 1857. Sharma & Kachroo, Fl. Jammu 1: 109. 1981; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 42. 2012; Bhellum, Fl. ASC. Campus, Srinagar (Kashm.) India 48. 2016.
Flower colour: Yellow
Flrs. & Frts.: May-October
Specimens examined: Mansar (400 m) Bhellum 25608
Distribution: India (Jammu & Kashmir), Semi-arid regions, Tropical and Subtropical regions

Sida alba L., Sp. Pl. ed. 2, 960. 1763. Masters in

Hooker f., Fl. Brit. India 1: 324. 1874. Sharma & Kachroo, Fl. Jammu 1: 110. 1981.
Flower colour: Yellow
Flrs. & Frts.: June-November
Specimens examined: Gurha (300 m) Bhellum 25016
Distribution: India (Jammu & Kashmir), Pakistan

S. cordata (Burm. f.) Borss. in Blumea 14(1): 182. 1966; Bhellum & Magotra, A Cata. Fl. Pl. Doda, Kishtwar and Ramban Districts (Kashm. Himal.) 42. 2012.
Flower colour: Yellow
Flrs. & Frts.: August-October
Specimens examined: Vijypur (300 m) 25070
Distribution: India (Jammu & Kashmir), throughout Tropical and Subtropical regions of the world.

S. spinosa L., Sp. Pl. 683. 1753; Masters in Hooker f., Fl. Brit. India 1: 323. 1874. Paul in Sharma *et al*., Fl. India 3: 292. 1993.
Flower colour: Yellow
Flrs. & Frts.: August-October
Specimens examined: Gurha and Baguna (350 m) Bhellum 25082
Distribution: India (Jammu & Kashmir), Pantropical

Urena lobata L., Sp. Pl. 692. 1753; Masters in Hooker f., Fl. Brit. India 1: 329. 1874; Sharma & Kachroo, Fl. Jammu 1: 111. 1981. Naqshi et al. in Ann. Miss. Bot. Gard. 75(4): 1522. 1988.
Flower colour: Pink
Flrs. & Frts.: August-October
Specimens examined: Rahya (300 m) Bhellum 25024
Distribution: India (Jammu & Kashmir), throughout Tropical and Subtropical regions of the world.

4. DISCUSSION

The taxonomic study of a particular region is of paramount importance as the floristic wealth is to be utilized for different aspects for national interests. Considering this fact, the present study was conducted in the current limits of district Samba of Jammu and Kashmir State. A total of 677 species representing 442 genera and belonging to 119 families of angiosperms (Table I) have been recorded. The Flora of this district is more similar to that of Flora of tropical region however, presence of some mixed elements of subtropical region and a few temperate species cannot be ignored. The ratio of genus to species is very low because of the small area of the district. The number of species of district Samba is less than that of district Udhampur merely because of the comparatively smaller area of the district. The district needs more attention to revise the families and genera of different families to investigate the raw wealth of this area. All the species are arranged in the alphabetical order in each family reported from this district. Comparison of the species, genera and families of the angiosperms in its immediate neighbourhood has been presented (Fig. 4).

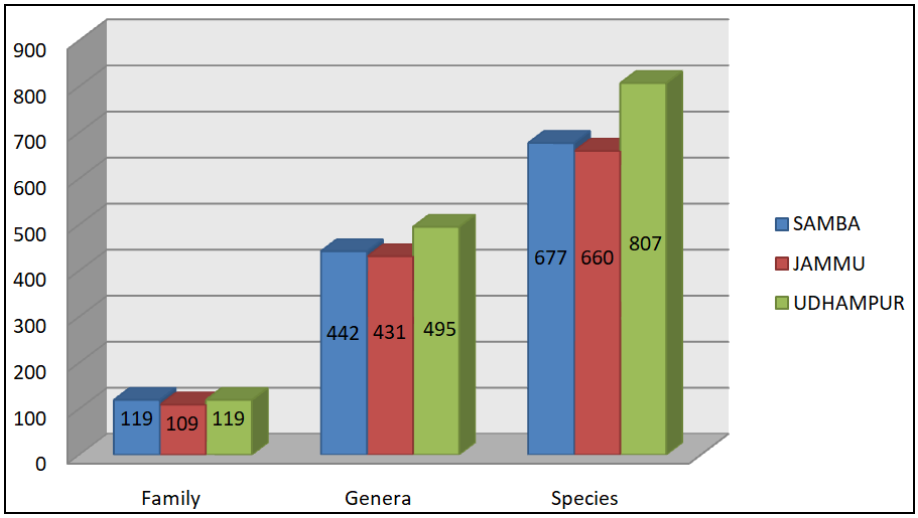

Figure 4. Bar Graph showing comparative account of the numerical size of families, genera and species in Samba, Jammu and Udhampur districts of Jammu and Kashmir State.

The highest number of species is presented in district Udhampur (807 spp.) [16] followed by district Samba (677 spp.) (Fig. 4) (author of current publication) and Jammu (660 spp.) with the lowest number of species [2].

Among the three families with a largest number of species are Poaceae, Fabaceae, Asteraceae in district Samba. Poaceae occupies the first place followed by Fabaceae and Asteraceae. This clearly indicates that number of species increases with the region from Tropical to Subtropical and Temperate zones. However, the most dominant family of flowering plants is Asteraceae in Alpine Flora of Kashmir Himalaya [12].

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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PLATES (A-F)

A. *Ranunculus laetus* Wall. ex Royle;

B. *Cissampelos pareira* L.

C. *Argemone mexicana* L.*;*

D. *Argemone ochroleuca* Sweet.

E. *Malvastrum coromandelianum* (L.) Garcke;

F. *Silene conoidea* L.

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Isolation and characterization of fungi isolated from Nigerian cocoa samples

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ABSTRACT

Ten fungal isolates were cultured from 40 samples of powdered cocoa by plating on Potato Dextrose Agar and incubation at 25°C for 7 days. They were subjected to macro- and micro-morphological examination to determine their genus and species. Some of the identified fungal species have been reported to produce industrially important enzymes, which they utilize to degrade a wide variety of substrates, while others have been reported to produce mycotoxins and also incite diseases in humans and animals. For the fungi identified, those that had the potential to produce mycotoxins were then analyzed using thin layer chromatography as well as ultra-pure liquid chromatography. Finally, we performed molecular analyses for our fungal isolates, to confirm or refute their morphological identifications, by amplifying and sequencing a portion of their respective ITS regions. Our findings supported most of the morphological identifications, but there were some observed inconsistencies based on mycotoxin and molecular examinations. Our findings underscore the importance of a multi-faceted approach to fungal identification. The following six genera or species were distinctly identified from the isolates: *Aspergillus flavus*, *A. niger*, *A. tamarii*, *A. violaceofuscus*, *Neocosmospora ramosa* and *Syncephalastrum racemosum*. The availability of these fungal isolates will serve as a reference point for researchers in southwestern Nigeria, thereby improving the quality and integrity of cocoa research in this region.

Keywords: Cocoa; Fungi; Mycotoxins; Nigeria; Ultra pure liquid chromatography.

1. INTRODUCTION

Although native to Central and South America, cocoa is now one of the major cash crops produced in Nigeria. It is used as a raw material for products such as cocoa powder, coffee, cocoa beverages and chocolates [1]. In the 1960s, cocoa was a major agricultural export crop and a critical foreign-exchange earner for Africa. Prior to the discovery of crude oil in commercial quantities in the 1970s, Nigeria was the world's second largest producer of cocoa. However, the average cocoa production fell from about 420,000 tons in the 1960s to 170,000 tons prior to the democratic dispensation of 1999. In the last several years, production levels have been maintained at or near 192,000 tons (2015 and 2016), making Nigeria the

sixth largest producer [2]. Because of its export value [3], it is essential that cocoa is produced and stored in the best condition to make contaminants, like fungi and bacteria, unattractive to the pods and seeds.

The major contaminants of cocoa are filamentous fungi, which infect cocoa beans and their seeds [4, 5]. Some of these cocoa-infecting fungi have been reported to produce mycotoxins [6, 7], which can pose a multitude of health risks to humans and animals when consumed. Previous mycological studies on fungal characterization from cocoa in Nigeria have adopted cultural methods alone [5, 8-11], which can result in fungal misidentifications. Information about the identification of fungi from cocoa using a combination of cultural and molecular methods is sparse in Nigeria. In Nigeria, cultivation of cocoa is regaining popularity, after many years of neglect, as reports surface that claim a global shortage of cocoa is possible, which is marketed raw or processed for local consumption and export. In order to safeguard the health of consumers, proper identification, leading to prescription of interventions against fungal contaminants, is necessary. The aims of this study are therefore, to characterize fungi of human importance from powdered cocoa using a combination of cultural and molecular methods, and to create a mini-culture selection center for filamentous fungi in Nigeria for future scientific references. This will confer integrity and credibility on mycological research.

2. MATERIALS AND METHODS

2.1. Fungal characterizations from powdered cocoa

Fungi were isolated from 40 powdered cocoa samples. The samples were cultured on Potato Dextrose Agar (PDA) and incubated at 25^{o}C for 7 days. After sub-culturing, pure fungal samples were sent to the Southern Regional Research Center (United States Department of Agriculture) in New Orleans for identification and analysis as earlier reported [7].

The powdered cocoa samples were also tested for the presence of several mycotoxins using Ultra-High Performance Liquid Chromatography (UPLC). Ground cocoa (1 g) was extracted with methanol (2 ml) for 24 h at room temperature. The extract was filtered, and the filtrate was then concentrated under nitrogen gas to dryness. Each extract was re-dissolved in methanol (to 5 mg/ml) and filtered through a Spin-X 0.22µm centrifuge spin tube filter (Corning® Costar®, Corning, New York). Standards for aflatoxins (B1, B2, G1, G2), cyclopiazonic acid (CPA), sterigmatocystin (ST), citrinin, deoxynivalenol (DON), and ochratoxin A (OTA) were used to develop methods for mycotoxin identification. The presence of aflatoxin B1 (rt = 4.37 min.), B2 (3.39 min.), G1 (2.78 min.), G2 (2.21 min.) and OTA (8.33 min.) were determined using a Waters Acquity UPLC system (Waters Corp., Milford, Massachusetts). Analysis involved subjecting each sample to 40% MeOH in water for 3 min., a gradient to 100% MeOH over 5 min., then 100% MeOH for 4 min. before re-equilibration to 40% MeOH in water for 5 min. on a BEH C18 1.7µm, 2.1 x 50 mm column. Aflatoxins and OTA were detected by fluorescence (ex = 365 nm, em = 440 nm and ex = 330 nm, em = 460 nm, respectively). The remaining mycotoxins investigated(CPA rt = 4.96 min., ST rt = 4.40 min., citrinin rt = 1.90 min., DON rt = 0.55 min.) were analyzed on the same system using 40% acetonitrile (0.1% formic acid) in water (0.1% formic acid) for 2.5 min., a gradient to 100% acetonitrile (0.1% formic acid) over 2 min., then acetonitrile (0.1% formic acid) for 6 min. before re-equilibration to 40% acetonitrile (0.1% formic acid) in water (0.1% formic acid) for 5 min., with photodiode array detection.

2.2. Characterization of sampled fungi

Morphological examinations of isolated fungi were performed as previously reported [7]. These required the use of an Olympus BH-2 microscope and dichotomous keys, and microscopic characters were photographed using a Nikon Digital Sight DS-Fi2 camera. Thin-layer chromatography (TLC) for aflatoxin and CPA detection, involving any putatively identified *Aspergillus* isolates, was also performed as previously reported from fungal cultures [7], molecular examinations involved amplification and sequencing of the internal transcribed spacer (ITS) region similar to Fapohunda et al. [7]. Some of the fungal isolates did not amplify with the

original ITS1 and ITS2 primers used previously, so new ITS primers were acquired that would amplify a larger portion of the ITS region (~600 bp). This new primer pair included a modified ITS1 forward primer (CTTGGTCATTTAGAGGAAGTA) and an ITS4 (TCCTCCGCTTATTGATATGC) primer as the reverse.

3. RESULTS AND DISCUSSION

We morphologically identified the sampled fungal species to be *Aspergillus tamarii* (SF 51, SF 54, SF 57) (Fig. 1); *A. niger* (SF50, SF 58) (Fig. 2); *Aspergillus flavus* (SF53, 55) (Fig. 3); *A. japonicus* (SF52) (Fig. 4); *Fusarium chlamydosporum* (SF56) (Fig. 5) and *Syncephalastrum racemosum* (SF59) (Fig. 6). Based on recent reports, the species name of *A. japonicus* is no longer valid and is now known as *A. violaceofuscus* [12]. Although *A. niger*, *A. oryzae* and *A. tamarii* have never been reported to produce aflatoxin, the isolates representing these species were subjected to mycotoxin analysis along with the *A. flavus* isolate. We detected the presence of aflatoxin for our *A. flavus* isolate (SF53), but we also found the *A. oryzae* isolate (SF55) produced a high concentration of aflatoxin B1. Therefore, we determined SF55 must have been misidentified and would require molecular examination to confirm or refute its morphological identification. None of the other isolates examined were found to produce aflatoxin. *A. niger* and *A. violaceofuscus* have the potential to produce ochratoxin A (OTA), but we were able to detect only a trace level of OTA for our *A. violaceofuscus* isolate (SF52).

Our molecular examinations of each isolate's ITS region confirmed the morphological identifications for most of the species sampled. BLAST query of the *A. oryzae* isolate's ITS region indicated higher homology with *A. flavus* sequences. This made more sense because of the production of aflatoxin B1 for SF55, which is believed to be impossible for *A. oryzae*. *A. oryzae* is considered a domesticated form of *A. flavus*, so morphologically they appear very similar. It took metabolic and molecular examinations to help us re-classify SF55 as *A. flavus*. Only a genome-wide analysis of SF55 would allow us to be certain of our re-classification. The only other conflicting species identification observed was for the *F. chlamydosporum* (SF56) isolate. Its ITS BLAST results indicated highest identity with an organism named *Neocosmospora ramosa*.

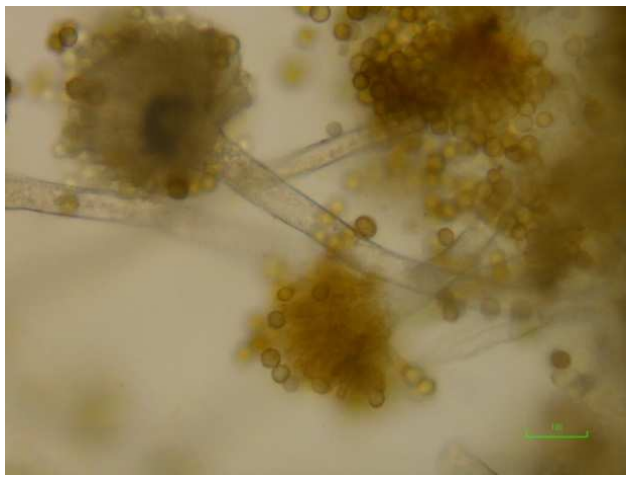

Figure 1. *Aspergillus tamarii* (SF51, SF54, SF57).

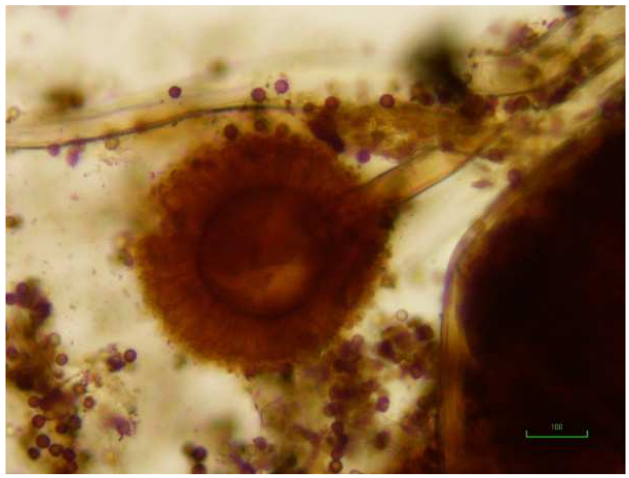

Figure 2. *Aspergillus niger* (SF50, SF58).

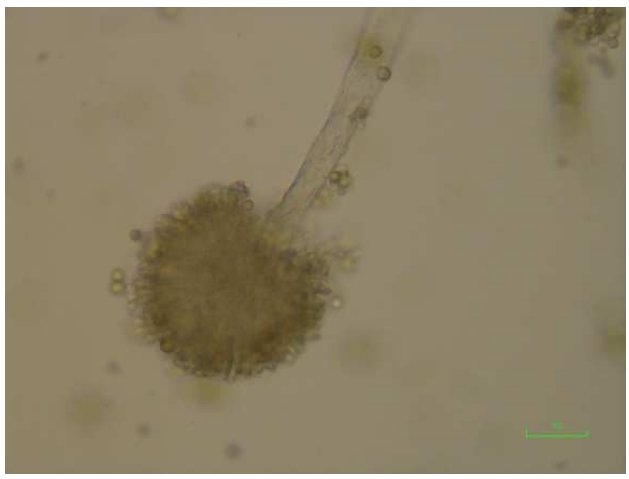

Figure 3. *Aspergilllus flavus* (SF53, SF55).

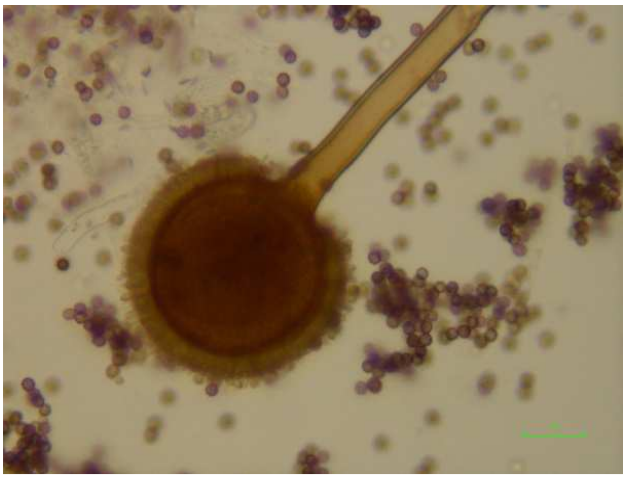

Figure 4. *Aspergillus violaceofuscus* (SF52).

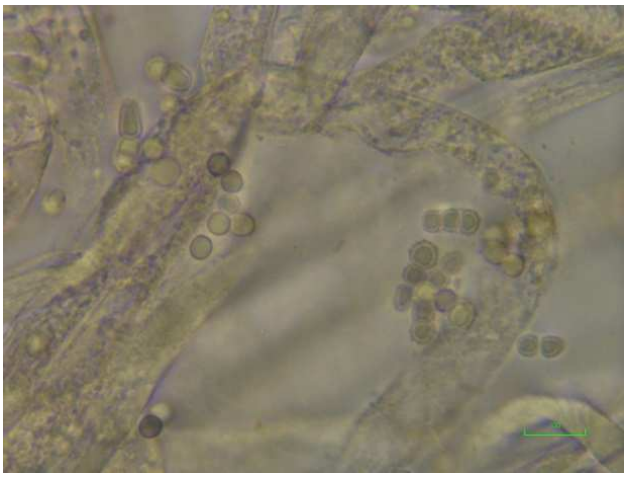

Figure 6. *Syncephalastrum racemosum* (SF59).

Figure 5. *Neocosmospora ramosa* (SF56).

This genus is considered *Fusarium*-like and similar to the teleomorphic genus *Haematonectria* [13]. Through recent phylogenetic inferences, it was determined that a 19th century genus name, *Neocosmospora*, was congeneric with the more recent genus name of *Haematonectria* [14-16], therefore, *Neocosmospora* has been reinstated as a sexual genus. Due to a lack of reports, it is uncertain whether or not *N. ramosa* poses serious health risks through pathogenicity or mycotoxicity. GenBank accessions for the ITS sequences of our 10 fungal strains are listed in Table 1.

Table 1. Molecular (ITS) identifications with accession numbers.

Strain	Molecular identification (ITS)	GenBank accession
SF50	*Aspergillus niger*	MG976495
SF51	*Aspergillus tamarii*	MG976496
SF52	*Aspergillus violaceofuscus*	MG682503
SF53	*Aspergilllus flavus*	MG976497
SF54	*Aspergillus tamarii*	MG976498
SF55	*Aspergilllus flavus*	MG976499
SF56	*Neocosmospora ramosa*	MG682504
SF57	*Aspergillus tamarii*	MG682505
SF58	*Aspergillus niger*	MG976500
SF59	*Syncephalastrum racemosum*	MG976501

Economic importance

A. tamarii has been reported to produce enzymes such as xylanase [17, 18], tannase [19] and protease [20] that are useful in the bioenergy and food industries.

A. niger has been reported to produce beneficial compounds such as amylase enzyme [21-23], as well as citric acid [24-26]. Some strains of *A. niger* have been reported to produce mycotoxins

such as fumonisin and ochratoxin [27]. It has also been reported to cause black mould disease in onions [28, 29].

A. flavus is most known for its ability to produce aflatoxins [7, 30, 31], toxic metabolites which have been rated as class 1A carcinogen by the International Agency for Research of Cancer [32]. It also produces a protease enzyme that may be useful to industry [33].

A. violaceofuscus (formerly *A. japonicus* according to Jurjevic et al. [12]) has been reported to produce fructo-oligosaccharides, which have the potential to improve food quality [34], as well as pectinase [35].

Although this is a fairly new species with scarce information in literature, it is a close relation of *N. vasinfecta* which was associated with the wilt of cotton, watermelon and cowpea [36]. Another not too distant species, *F. chlamydosporum*, has been reportedly used for bioremediation of wastewater [37] and production of antimicrobial agents [38]. It also has potential to infect agricultural products such as wheat and cassava chips [39, 40].

S. racemosum has been reported to cause various diseases in humans [41-43].

4. CONCLUSION

There are many fungi of industrial, medical and agricultural importance in Nigeria, and yet a mycological culture collection center is still lacking. The present study will therefore serve as a component of the proposed mycological map involving Nigerian crops apart from giving insight into a likely cause of cocoa-borne morbidities in humans. Fungal contaminants of cocoa seeds have resulted in spoilage and wastages for this crop that once served as a critical foreign exchange earner for Nigeria, particularly in the 1960s. Export values of agricultural commodities had decreased due to the detection of mycotoxins at levels beyond global and EU standards. Sometimes reactions on food contaminants, from importing countries could include rapid alerts, rejects and trade bans. The availability of these identified fungal isolates will serve as a reference point for researchers in Nigeria, thereby improving the quality and integrity of food-related research in this region. Our study has also illustrated the importance of a holistic method of fungal identification that involves morphological, metabolic and genetic examinations. Fungal misidentifications may result in incorrect assessmants; and subsequently, ineffective interventions. The more accurate our identifications, the better our chances at minimizing risks posed by harmful fungal pathogens.

AUTHORS' CONTRIBUTIONS

FSO and ASO initiated and designed the study. FSO, AKI, ADE and OSK isolated and carried out preliminary identification of the fungi. Morphological and molecular characterization (with illustrations) and mycotoxin analysis were done by MGG. FSO, MGG, AKI, and ASO wrote and edited the manuscript. All authors read and approved the final manuscript

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Research Article

Effect of hormone for *in vitro* propagation of *Asparagus racemosus* Wild.

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ABSTRACT

Asparagus racemosus Wild. is an undershrub, climber which is extremely branched with woody stem and spines like reduced leaves. It founds in tropical and sub-tropical region of Nepal up to 1500 meter above sea level. *A. racemosus* Wild. is an endangered species widely used in modern drug development and Ayurveda as well. The subculture explant shows responses to the different concentration of hormone. The composition of MS + 0.5 mg/l BAP and MS + NAA 0.5 mg/l + kinetin 1 mg/l most significant for shoot multiplication. Over them, the composition MS = 0.01 mg/l NAA + 1.0 mg/l BAP along with MS + 0.5 mg/l NAA = 1.5 mg/l kinetin is effective for the development of shoot in *Asparagus racemosus*. The hormone play important role on the regeneration of shoot *in vitro* condition. The knowledge of hormonal requirement help to promote the development and growth of endangered plants for their rapid propagation.

Keywords: *Asparagus racemosus* Wild.; 6-benzyl amino purine (BAP); Growth and development; *In vitro* propagation; Medicinal plant; 1-napthalene acetic acid (NAA).

1. INTRODUCTION

Nepal has a wide range of climatic variation due to its geography and topography and almost all types of climate, season and soil from the world [1] that have already been identified [2]. The Ayurveda (homeopathic) health care system depends on the use of these highly valued native medicinal plants. Some medicinal plant from Kathmandu valley [3] from Argakahaci district [4]; recently reported from Biratnagar, eastern Nepal [5]. The first attempt to regenerate plant from tissue reported [6]. The culture medium [7, 8] developed for tobacco has served as the starting media for many plants.

Kohmura et al. [9] have developed an effective micro-propagating system involving induction of multiple bud clusters and somatic embryogenesis in *A. officinalis* L. CV "Hiroshima green", and other 14 genotypes. Schröder and Eimert [10] have studied the temporary immersion of *in vitro* grown internodal pieces of *A. officinalis* and their incubation in the dark in a liquid embryo induction medium. The explant can be very small pieces of plants, such as embryo, seed, shoot tip, meristem, root tip, callus, single cell and pollen grain [10].

Gupta et al. [11] tested seed germination of *A. racemosus* Wild. They observed a wide variation in germination percentages of the seeds ranging from 17 to 60 in different collection samples. Saensouk and Suddee [12] used young leaves and nodes of *A. racemosus* as explants and MS as the basal medium, NAA, and BA as the PGRs in their experiment. The percentage of callus on medium

combination with 0.5 mg/l NAA and 2 mg/l BA were 90 percent and 80 percent from leaves and nodes, respectively. Shooting was 100 percent from leaves cultured on medium added with 1 and 2 mg/l BA. Pontaroli and Camadro [13] studied the callus growth and plant regeneration from long-term callus cultures in two clones of *A. officinalis* cv. Argenteuil, to establish a suitable protocol for a prospective *in vitro* selection program.

Bopana and Saxena [14] have pointed out the importance of *A. racemosus* Wild. as an important medicinal plant of tropical and subtropical India. They have listed the medicinal usage of this species like treatment of neurodegenerative disorders and in alcohol abstinence-induced withdrawal symptoms.

Dutta [15] has mentioned the uses of *Asparagus racemosus* in treatment of various disease of liver, scalding, urine, gleets, and gonorrhea. Root power given as tonic for strength. Bopana and Saxena [16] mentioned that due to destructive harvesting, the natural population of *A. racemosus* is rapidly disappearing, and it recognized as 'vulnerable'. Hurgoiu and Blidar [17] studied the callus growth and organogenesis from the previously induced callii in *A. officinalis* (Jersey Knight F1).

Kumar and Vijay [18] used MS medium and BA and K as cytokinin (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) and IAA, IBA and NAA as auxins (0.1, 0.5 and 1.0 mg/l) in combinations to see the various effects in *A. racemosus* nodes. They very little callus with IAA 3.0 mg/l whereas maximum callus as well as shoot proliferation at 2.0 mg/l NAA. They observed that BA up to 2.0 mg/l with any concentration of IAA either a very little or no callus at all. Pant and Joshi [19] have successfully multiplied *A. racemosus* using tissue culture technique. Plant growth regulators are important in plant tissue culture since they play vital roles in steam elongation, tropism and apical dominance. Auxins, cytokinin, gibberellins, ethylene and abscisic acid are the five general classes of hormones [20].

The objectives of this research are to find out a reliable and cost effective method of rapid propagation of the plant. *A. racemosus* as well as to understand its behavior under different phytohormone conditions.

2. MATERIALS AND METHODS

2.1. Plant material

The younger plant material (*Asparagus racemosus* Wild.) collected from botanical garden of Department of Botany of Tri Chandra campus, Kathmandu.

2.2. Surface sterilization of *ex-plant*

Shoots washed under running tap water for about one hour with few drops of liquid detergent. After washing with detergent the explants thoroughly rinsed with distilled water for 4-5 times to remove any traces of detergent remaining in explants. After these treatments, explants taken inside the laminar airflow for further sterilization. Explants were surface sterilized with freshly prepared 0.1% w/v aqueous solution of Sodium hypo chloride for 10 minutes. Then again, explants thoroughly rinsed 3-4 times with sterilized distilled water to remove any traces of Sodium hypo chloride.

2.3. Sterilization of glassware's

All the glassware dipped in the detergent water for overnight. They washed thoroughly with tap water using bottlebrush then autoclave.

2.4. Sterilization of media and equipment

All the media, utensils (forceps, blade holders, blades if reused, needles, brush etc.) and sterile water used inside the laminar hood were sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

2.5. Preparation of *ex-plants*

For the explants, after mentioned surface sterilization processed used. In addition, they used as the explants for *in vitro* experiments. In case of shoot tip, culture approximately 0.5-2.0 cm long parts of these organs excised inside the laminar hood using a sterile blade.

2.6. Media preparation

A medium is the formulation of different inorganic salts and organic compounds necessary for the nutrition of plant or plant part under *in vitro* culture condition. There are various types of culture media recommended for various types of cultures and plants. This is necessary to identify a suitable media for better results (outcomes). This can done either by a better understanding of the nutritional requirements of cultured cells and tissues or by the results of previous similar experiments conducted by various workers on similar plants or plant parts. In general, the tissue culture medium must contain the 16 essential elements for plant growth.

2.7. Preparation of stock solutions

Different stock solutions containing macro elements (A), microelements (B), iron source (C), vitamins (D) and iodine sources (KI).

The strength of "Stock A" and "Stock C" containing macro elements raised to 10x while making the stock solutions for convenience. All the compounds of macro salts dissolved in distilled water and stored in the brown bottle inside the refrigerator. The iron source compounds needed slight warming to dissolve completely in the distilled water.

Similarly, the strengths of the micronutrients and vitamins raised to 100x in the stock solutions. The KI solution was prepared separately.

For the preparation of stock solutions, all the chemicals were weighed separately using a digital electronic balance. The individual chemicals dissolved one by one and a required final volume (500 ml) made by adding required amount of distilled water. All the stocks were stored in clean brown bottles inside the refrigerator.

The myoinositol added freshly during the media preparation time.

2.8. Preparation of MS medium

For the preparation of one liter of MS medium, following procedure followed. By using either a measuring cylinder or a pipette 100 ml of stock A (x10), 1 ml of stock B (x100), 1 ml of KI (x100) solution, 10 ml of stock C (x10) and 1 ml of stock D (x100) were mixed in a conical flask of capacity 1000 ml. An appropriate amount of myoinositol (0.1 g) weighed and added freshly. Similarly, 30 g of sucrose added and stirred with the help of a glass rod. When the sucrose completely dissolved, the pH measured using a pH meter. The pH of the medium adjusted to 5.8 ±0.1 with 0.1 M HCl or NaOH. After adjusting the pH, the medium heated over an electric heater. When the media was nearly boiling took out of the heater and added pre-weighed 8.00 g of agar with continuous stirring.

Now, the media was again heated, boiled and poure in glass jam bottles. One liter of medium poured in 20 jam bottles. The mouths (openings) of the bottles were covered. All the media sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

The required amounts of PGR/s in the media added before autoclaving from their stocks using sterile pipettes.

2.9. Preparation of PGRs stock solutions

For the preparation of hormone solutions of all the auxins and cytokinins, 10 mg of each of the PGRs dissolved in few drops of their respective solvents (ethanol, KOH and NaOH) in separate test tube. These solutions made 100 ml by adding distilled water. These taken as the stock solution of 100 mg/l and are preserved in brown bottles in the freezer. The amount in ml of the stock added to one liter of the final medium makes the same amount of PGR in mg/l (i.e. 1.0 ml of NAA stock if added to one liter of medium makes 1.0 mg/l NAA).

For the preparation of different concentrations of different hormones from the stock solutions for various purposes, the following formula used:

$$S_1V_1 = S_2V_2$$

Where: S_1= strength of stock i.e., 1000 ppm., V1= volume of the stock to be taken, S_2 = strength of the hormone required (ppm), V_2 = Total volume required.

2.10. Inoculation of *ex-plants*

All the media and necessary utensils were, again sterilized under the UV light for 45 minutes before using. The laminar airflow hood washed/

sprayed with alcohol before turning the UV light on to minimize the risk of contamination. The explants after a series of surface sterilization process mentioned were ready for inoculation. The explants of about 0.5-2.0 cm were prepared and inoculated in the medium under the laminar airflow hood. The transfer was done close to a burner with a pair of sterile forceps. Similar method used in all cultures and sub cultures.

2.11. Culture conditions

All the culture bottles containing specific media after inoculation of the explants kept in a culture room. The temperature of the culture room maintained at 25±2^{0}C by running an air conditioner. The room was illuminated for 16-hours (everyday photoperiod) with the light intensity of 3000 lux using cool white fluorescent tubes.

2.12. Acclimatization

The *in vitro* rooted plantlets removed, washed carefully and planted in the coco peat. All the rooted plantlets (both *in vitro* and *in vivo*) transferred to sand soil mixture (1:1). All the plantings were regularly observed, and watered at an interval of 2-5 days depending upon the moisture on the bed. The humidity inside the dome maintained up to 90% for the first week. Gradually the humidity decreased by allowing more air to circulate in the successive weeks. Finally, established plants either transferred to the garden or distributed to the interested growers.

3. RESULTS

3.1. Culture of shoot tip of an *ex-plant*

For the inducing of multiple shoots, shoot tips of early young shoot of *A. racemosus* Wild. was inoculated on the MS basal media, supplement with various pairs of concentration of growth hormones (NAA, BAP, kinetin). 5 weeks of first inoculation, first sub culture done slightly changing the initial pairs of hormone concentrations, and it was remained for about 6 week (Figs. 1 and 2).

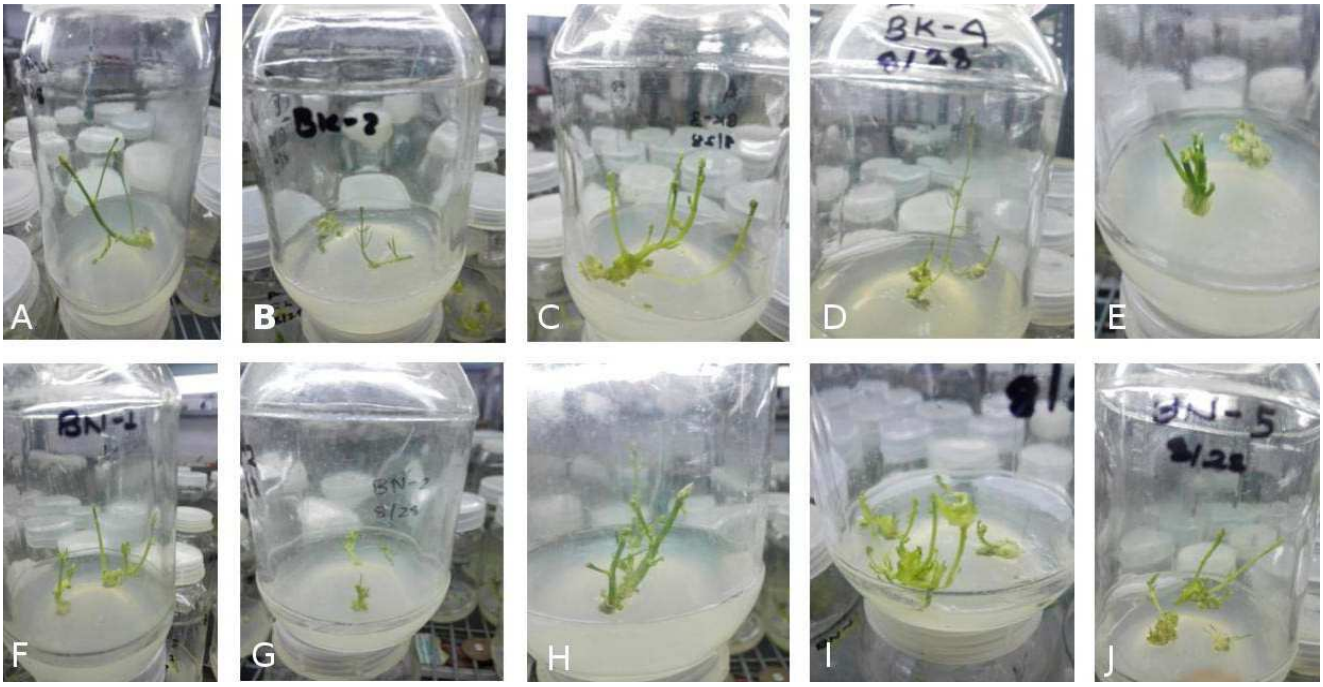

Figure 1. Four-week-old plant in MS media: **A.** NAA 0.1 mg/l + kinetin 0.1 mg/l, **B.** 0.1 mg/l NAA + 0.5 mg/l kinetin, **C**. NAA 0.5 mg/l + Kinetin 1.0 mg/l, **D.** NAA 0.5 mg/l + kinetin 1.5 mg/l, **E.** NAA 0.5 mg/l + kinetin 2.5 mg/l, **F.** NAA 1 mg/l + BAP 1 mg/l, **G.** NAA 0.1 mg/l + BAP 1.0 mg/l, **H.** NAA 0.01 mg/l + BAP 1 mg/l, **I.** NAA 0.1 mg/l + BAP 0.5 mg/l, **J.** NAA 0.01 mg/l + BAP 0.5 mg/l.

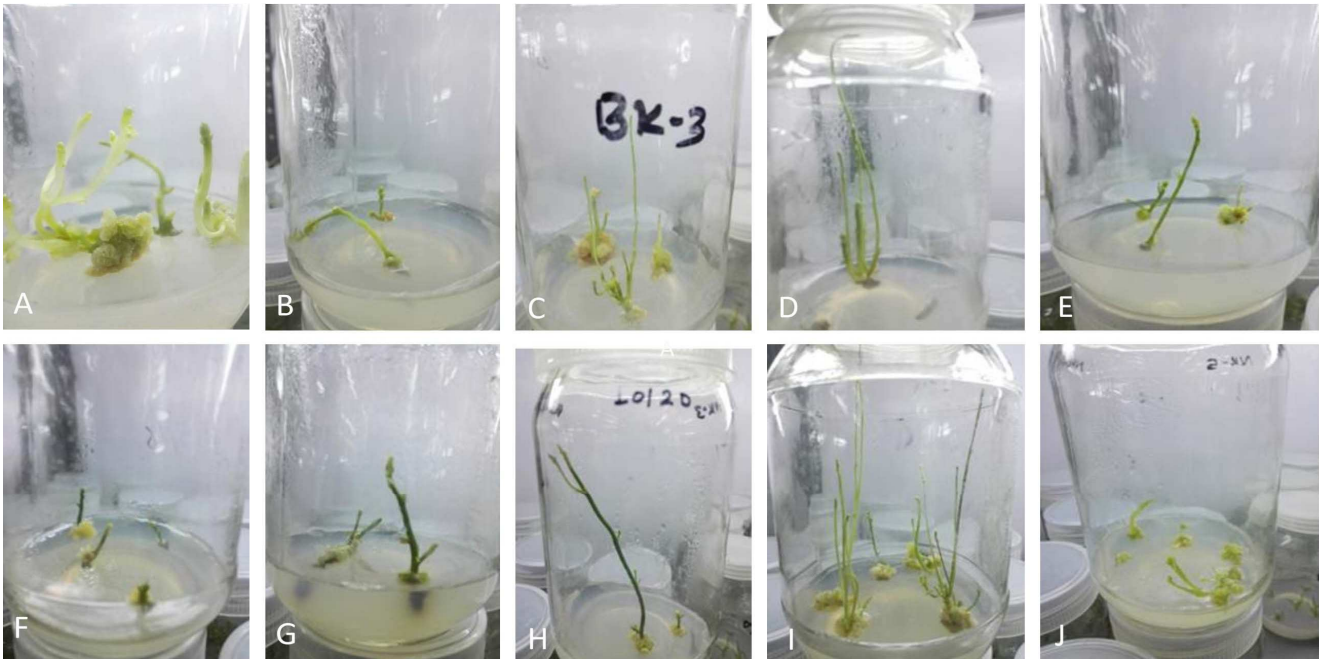

Figure 2. Five-week-old plant in MS media: **A**. NAA 1.0 mg/l + kinetin 0.1 mg/l, **B**. NAA 1.0 mg/l + kinetin 0.5 mg/l, **C**. NAA 0.5 mg/l + kinetin 1 mg/l, **D.** NAA 0.5 mg/l + kinetin 1.5 mg/l, **E**. NAA 0.5 mg/l + kinetin 2.5 mg/l, **F**. NAA 0.1 mg/l + BAP 0.1 mg/l, G. NAA 0.1 mg/l + BAP 1.0 mg/l, **H.** NAA 0.01 mg/l + BAP 1 mg/l, **I.** NAA 0.1 mg/l + BAP 0.5 mg/l, **J.** NAA 0.01 mg/l + BAP 0.5 mg/l.

3.2. Effect of different hormone concentrations (Tables 1-4)

Table 1. Effect of kinetin and NAA (5 weeks of inoculation).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	Kinetin				
1	0.1	0.1	Shoots + callus	5-6 cm	3	Well growth of shoot, callus at the base of the shoot
2	0.1	0.5	Shoots + callus	4-5 cm	3	Callus appears at the base of the shoot. Normal growth of shoot
3	0.5	1	Shoots + roots	5-6 cm	5	Well growth of shoot. Multiplication of the shoot, root develops
4	0.5	1.5	Shoots	6-7.5 cm	4	Well growth of shoots
5	0.5	2.5	Stunted shoots	2-3 cm	5	Growth of shoots stops after 2-3 weeks. Small mass of callus seen

Table 2. Effect of NAA and BAP (5 weeks of inoculation).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	BAP				
1	1	1.0	Shoots + callus	3-4 cm	4	Well growth of the shoot, small mass of callus at the base of shoot
2	0.1	1.0	Shoots + callus	2-3 cm	3	Mass of callus at the end of shoot tip
3	0.01	1.0	Shoots	3-4 cm	5	Well growth and multiplication of the shoot
4	0.1	0.5	Shoots + root	3-4 cm	6	Shoot multiplication and well growth of the shoot + root development
5	0.01	0.5	Shoots	4-5 cm	5	Well growth of the shoot

Table 3. Effect of NAA and kinetin (6 weeks of subculture).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	Kinetin				
1	1	0.1	Callus	-	6	No any significance change in the shoot development, small mass of callus at the end
2	1	0.5	Shoots + root	1-2cm	2	Growth rate of shoot is low, small hairy root appears
3	0.5	1	Well grow of Shoots + roots	5-6 cm	10	Well growth of shoot + root
4	0.5	1.5	Shoots only	6-7.5 cm	5	Well growth and multiplication of the shoots. Growth of roots
5	0.5	2.5	Stunted shoots only	2-3 cm	4	Growth of shoot stops suddenly

Table 4. Effect of NAA and BAP (6 weeks of subculture).

S.N.	Growth hormones		Growth response	Height of shoots	Number of shoots	Observation
	NAA	BAP				
1	0.1	0.1	Shoots + callus	2-3 cm	3	Extensive mass of callus along with developing new shoots
2	0.1	1.0	Shoots + callus	3-4 cm	4	Shoot growth + callus
3	0.01	1.0	Shoots + callus	5-6 cm	4	Well growth of shoot and mass of callus at base of shoots
4	0.1	0.5	Shoots + roots	8-9 cm	13	Growth and development of shoot along with minute roots
5	0.01	0.5	Shoots only	3-4 cm	7	Growth of shoot, no callus

3.3 Acclimatization

After one months of hardening, these plantlets carried out for sand rooting. Sand rooting was done with the help of root hormone i.e. auxin. The survival rate of these plantlets were satisfactory i.e. out of 58 plantlets 31 (53.45%) plantlets were survived when these plants were transferred to the poly bag the survival rate was outstanding (Table 5, Fig. 3). Almost all the plantlets with root survived in polybag.

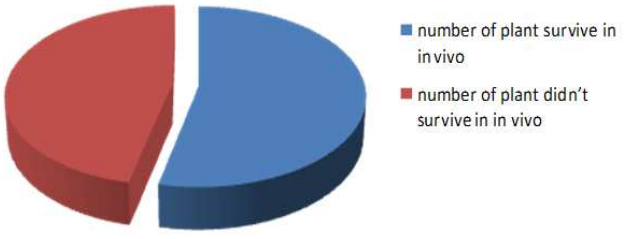

Figure 3. Pie chart showing the plants survive in *in vivo* condition.

Table 5. Plants showing response in *in vivo* condition (Green House).

Total no of plants	Plant survived *in vivo*	Plant can't survived *in vivo*
58	31	27
%	53.45%	46.55%

4. DISCUSSION

In vitro propagation of *A. racemosus* Wild. through the shoot tip of the plant successfully done in different hormone concentration of auxin and cytokinin. In this experiment, the growth of shoot was found to be normal than the growth of root.

Shrivastana et al. [20] working on *Citrus vulgaris* observed the requirement of BAP in shoot formation. However, in present experiment the rapid growth of shoots and multiplication of shoot observed in MS + NAA (0.1 mg/l) + BAP (0.5 mg/l).

Kumar and Vijay [18] reported successful establishment of plant raised through shoot tip

culture of *A. racemosus* Wild. for rapid clonal propagation. Shoot tip ex plant culture on the MS + kinetin (3.0 to 5.0 mg/l) reported multiple proliferations of shoots from the explant. In this experiment, multiple proliferations of shoots with growth of root observed in MS + NAA (0.5 mg/l) + kinetin (1.5 mg/l). The growth of root is due to the presence of auxin and kinetin-containing medium shows the growth of regenerated shoots.

Ranjitkar and Saiju [21] obtained micro shoot on BAP (3 mg/l) + NAA (0.1 mg/l) by shoot tip culture of *Rauwoflia serpentine* and subculture of these shoot tip on lower concentration of BAP (1 mg/l) + NAA (0.1 mg/l) results in shoot proliferation. Similar result obtained in this experiment; multiple numbers of small shoots obtained in MS + NAA (0.01 mg/l) + BAP (1 mg/l).

Ranjitkar et. al. [22] growth of shoots on MS + BAP (0.5 mg/l) + NAA (0.01 mg/l), and growth of multiple number of shoots in MS + BAP (1 mg/l) + NAA (0.01 mg/l), while culturing of shoot tip of *Swertia ciliata.* Similar result obtained in present experiment in same hormone concentration of NAA and BAP.

In combination of auxin and cytokinins maximum numbers of shoots were obtained in the hormone concentration of MS + NAA (0.5 mg/l) + kinetin (1.5 mg/l). On increasing the concentration of NAA and decreasing the concentration of kinetin, shoot didn't give any response, however callus developed at the end of the shoot i.e. in MS + NAA (1 mg/l) and kinetin (0.1 mg/l). higher concentration auxin accumulation might have affected in proper growth of the shoots because high concentration of auxin increase the production of ethylene; production and ethylene accumulation in the cultural media and may inhibit the growth and development of plant in *in vitro* culture. Conversely, ethylene may affect the transport and metabolism of the cytokinins, similar result with Rajkarnikar et al. [21] while doing shoot tip culture of *A. racemosus*. Paudel et al. [23] established hormonal effect on shoot regeneration for *Lycopersicon esculentum* L. in 11-12 week. Pant and Joshi [19] observe that BAP played good roles in shoot and bud inductions, whereas combinations of NAA and BAP at various levels were found to be effective in almost all cases, same result was obtained in present study. Also Aryal et al. [24] uses the hormonal treatment for the meristem culture in *Amomum subulatum* for elimination of Chhirkey and Foorkey.

Presence of kinetin along with the relatively lower concentration of NAA results in the growth and development of shoot, multiplication of shoots and at the end of the shoot, mass of callus obtained. In all the combination of auxin and cytokinin, none of the concentrations combine found to be statically significant to each other in inducing the shoot, multiplication of the shoot, callus development similar to that of Shrivastava and Rajani [20]. However in two pair of concentrations of auxin and cytokinin i.e. [NAA (0.5 mg/l) + kinetin (1 mg/l) and NAA (0.5 mg/l) + kinetin (1.5 mg/l)], among all the concentration combination only these two combinations were slightly statically significant in inducing multiple shoot. From this experiment, NAA at lower concentration along with high concentration of kinetin found to be greatly favored the shoot multiplication. This finding was similar to that work of the Pant et al. [19] with their experiment in the *A. racemosus.* Equal hormone concentration of NAA (0.1 mg/l) and kinetin (0.1 mg/l) favors the induction, growth and development of shoot, similar result obtained in NAA and BAP each of concentration of 1 mg/l. However, in case of very low concentration of auxin and NAA and high concentration of kinetin result is slightly different. The development of shoot at high concentration is not normal, growth of the shoot stunted after 2-3 weeks of inoculation.

From this experiment it was found that the most of the concentration combinations of NAA along with BAP were negatively significant in case of shoot elongation i.e. they retarded the growth of shoots but with KN the result was slightly better when KN or both were at higher levels. Although in some cases vitrifications were observed, higher Kn concentration generally gave shoot elongation whereas BAP mostly showed vitrification.

5. CONCLUSION

Due to lack of proper conservation strategies, technique, policies, awareness program, poverty, habitat destruction, illegal transport, over exploitation these plants are in state of extinction. In such a way *A. racemosus* Wild. is also in state of extinction.

From the overall study, it concluded that the plant tissue culture (i.e. *in vitro* propagation) technique is the most suitable technique for conservation of medicinal plants. It is easy method for production of large number of plant species within short period of time and in small place, without losing their properties and without damaging their original plant. Once suitable protocol is established, the cost will greatly reduce. From this experiment, we can conclude that MS + NAA 0.5 mg/l + kinetin 1.5 mg/l and MS + NAA 0.1 mg/l + BAP 0.5 mg/l is good for multiplication and development of shoots. In addition, can be concluded that micropropagation is the easiest, fastest and reliable method for the multiplication of this plant and other plants. With this technique, we can conserve the biodiversity for the present and future generation.

AUTHORS' CONTRIBUTION

NP: Manuscript preparation and experimental design. MRA: Experiment design, guidance, and RHP: Experiment design and data collection. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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