Development of tissue culture system for \textit{in vitro} regeneration, biochemical and molecular characterization of ginger varieties grown in Sikkim

AN ABSTRACT (THESIS)

SUBMITTED TO GUWAHATI UNIVERSITY
FOR THE DEGREE OF DOCTORS OF PHILOSOPHY IN (DEPARTMENT) IN THE FACULTY OF TECHNOLOGY

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Year 2013

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ABSTRACT

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The present work contains the experimental studies on four different aspects of ginger grown in Sikkim state of India. These aspects included the morphological and ecological evaluation on the ginger cultivars, standardization of tissue culture protocol for micropropagation of ginger, evaluation of antioxidant and antimicrobial properties and the studies of genetic variability of the cultivars as well as genetic fidelity of the micropropagated plants using RAPD markers.

Ecological studies on different ginger cultivars in Sikkim Himalaya using Geographic Information System (GIS) and overlay technique based on elevation and district wise study revealed that about 35.86% of ginger farmers of Sikkim preferred to cultivate cv. Majhauley followed by Gorubthangey (21.51%), Bhaisay (18.14%), Charinangrey (13.92%) and Jorethangey cultivars (10.97%). Out of a total of 237 plots of ginger growing areas, 125 plots were found without disease and 112 plots were found with fungal and bacterial diseases infection. No infection was recorded from north Sikkim, and cv. Charinangrey and cv. Majhauley were found less susceptible against pathogenic microorganism.

A total of sixteen morphological traits were examined that included five qualitative characters (growth habit, shoot intensity colour, colour of the bract tip of fully developed spike, leaf intensity of green colour and rhizome shape) and eleven quantitative characters. Enumerations of morphological characters on five cultivars based on quantitative traits found that plant height, numbers of shoots, shoot diameter, canopy, leaves density, leaf length, numbers of tillers rhizome thickness and weight of rhizome are statistically highly significant (P <0.05) and qualitative traits like spike bract tip colour and shape of rhizome, above all characters were important traits for differentiating among five ginger cultivars.
Correlation studies between mean weights of ginger was found directly related with mean plant height, mean canopy per plant, mean number of shoots per plant, mean shoot diameter per plant and mean number of leaf density per plant.

Other morphological characters found positively correlation between mean numbers shoots and mean number of leaf density per plant, mean leaf length per plant and mean spike length per plant these traits would directly improve rhizome character and yield in ginger. First three principal component analysis showed relatedness among the five cultivars based on ten quantitative and three qualitative morphological characters, of total 51.78 % variances were found. Morphological characters like plant height, canopy, numbers of shots, leaf density, spike length and shoot diameter, rhizome shape, spike length and leaf density and rhizome size played a significant role in differentiating character to discriminate between the five cultivars of *Zingiber officinale*.

An efficient micropropagation method was standardized for three cultivars of ginger (*Zingiber officinale* Rosc) namely cv Majuley, Bhaisay and Charinangrey. MS media with 3% of sucrose have been used with different concentration of various hormones. The maximum number of shoots and roots were observed on GM-23 (growth medium-23) that contained MS+ sucrose (30g/l), BAP (2.5mg/l), NAA (0.5 mg/l) activated charcoal (2mg/l).

The maximum number and mean length of shoots, roots and leaves were recorded on MS medium containing 2.5mg/l BAP and 0.5mg/l NAA with shoots numbers and lengths 19.98 ± 0.01 and 4.09 ±0.04, root number and length were 06.78 ± 0.21 and 05.53 ±0.05, and mean number of leaves 06.96 ±0.03 per explants and second was 3mg/l BAP and 0.5mg/l NAA with mean shoots number and length were 17.88 ±0.11 and 4.97±0.07, mean root length and number were 06.86 ±0.02, 05.56 ±0.02 and number of leaves was 06.55 ±0.01. After individually sub-cultured on same fresh medium, adventitious buds were initiated after 10–15 days of culture, complete plantlets developed within 40 days and maintained for 18 months without decreasing the multiplication rate. The success rate of transfer of *in vitro* grown plantlets to field condition was found to be 100%. Investigation was carried out for the determination of genetic conformity of micropropagated plants of *Zingiber officinale* cv Majhauley using Random amplified polymorphic DNA (RAPD) technique. Total fifteen different
arbitrary decamers were used as primers to amplify DNA from in vitro plant material to assess the genetic fidelity of 18 months old in vitro cultured plantlets. Total 38 amplified reproducible bands were produced from 10 primers with an average of 3.8 per RAPD primer, amplified bands were ranging from 150-950 base pair. All the primers were found to be monomorphic and no genetic variation was detected within the micropropagated plants. Present protocol may be used for testing of ginger propagules to ensure mass production of disease free planting materials.

Twenty five different extracts from five different ginger cultivars of Sikkim were investigated for their antibacterial activity against two Gram positive \textit{(Staphylococcus aureus} (MTCC 96), \textit{Bacillus subtilis} (MTCC 441)), and three Gram negative bacteria namely, \textit{Klebsiella pneumoniae} (MTCC 432), \textit{Escherichia coli} (MTCC 739), and \textit{Pseudomonas aeruginosa} (MTCC 424) using disc diffusion assay. Out of these, \textit{E. coli} (MTCC 739) did not exhibit susceptibility against any solvent extracts.\textit{Staphylococcus aureus} (MTCC 96) was most susceptible to chloroform extract of \textit{cv-Majhauley}, which showed widest zone of inhibition of 26 mm. The factor responsible for the most susceptible action of chloroform and ethanol extract may be accredited to the presence of active components responsible for antimicrobial activity. Statistically significant difference were achieved among all microorganism (P<0.01790), gram positive bacteria (P<7.69778E-15) and gram negative bacteria (P<1.06101E-09) studied. Furthermore an inversely significant correlation between total phenol content and antimicrobial activity were recorded against microorganism \textit{Staphylococcus aureus} (R²=-0.53, P<0.001) and \textit{B. Subtilis}(R²=-0.58, P<0.001) and presence of total flavonoids content in different cultivars of ginger showed an inversely significant correlation between microorganism \textit{Staphylococcus aureus}(R²=-0.75, P<0.001) \textit{B. Subtilis}(R²=-0.71, P<0.001). This study confirmed the presence of high antibacterial activity in ginger cultivars of Sikkim.

In antioxidant study, total phenolic and flavonoid contents as well as antioxidant properties of five different solvent (petroleum ether, ethanol, chloroform, methanol and acetone) extracts of rhizomes of five different cultivars of \textit{Zingiber officinale} were investigated. Evaluation of antioxidant activity was conducted using DPPH assay, Ferric-Reducing power assay and H₂O₂ scavenging activity. The results showed the total phenolic and flavonoid contents were in the ranged between 99.00 -228.557 mg/ml
GAE (Gallic acid equivalent) per gram and 45.12 mg/ml-621.52 mg/ml QE (Quercetin equivalent) of plant extracts respectively. The highest DPPH scavenging activity recorded in Acetone extracts of Majhauley cultivar, FRAP showed highest activity in chloroform extracts of Charinangrey and methanol extracts of Bhaisay cultivars showed highest H$_2$O$_2$ scavenging activity. All the extracts showed strong antioxidant activity comparable with or higher that of standard. As a conclusion, gingers found in Sikkim investigated in this study can be developed as natural antioxidant agents.

Genetic variations were studied on five cultivars of *Zingiber officinale* of Sikkim Himalaya using RAPD markers. A total number of 40 accessions collected from different eco-climates zones of Sikkim, India. A total of 63 RAPD primers were screened, out of which 21 primers showed consistent bands to evaluate polymorphism. A total of 104 clear and reproducible and scorable RAPD fragments ranging from 150-13000 bp were generated from 21 primers. Of the 104 scorable RAPD bands, 99 were found polymorphic. The amplifications products ranged from two (2)- thirteen (13) for different primers. Each primer produced on an average 4.5 polymorphic bands per primer. UPGMA method was used to construct dendrogram and the genetic relationships were determined among the five different cultivars of *Zingiber officinale* of Sikkim using Nei’s genetic distances. Cluster analysis of data using UPGMA algorithm placed the 40 accessions into three main clusters, the genetic dissimilarity matrix between genotypes ranged from (0.74994 - 5.33209) between the 5 cultivars studied. All samples showed three main clusters, viz., (1) Cluster I representing Charinangrey, Gorubthangey and Majhauley, Cluster II representing Jorethangey and Cluster III representing Bhaisay. The clusters revealed the level of relatedness (0.74994 -6.26042) between the 5 cultivars studied. Dendrogram was constructed using NTSYSpc based on RAPD markers, indicating the genetic diversity between five cultivars of ginger using a Jaccard’s coefficient. Cluster analysis of data using 40 accessions into four main clusters the genetic with a range 0.35-0.87 and the principal component analysis placed the 40 accessions into four groups. The results showed the prevalence of a relatively high level of polymorphism in the cultivars of ginger from Sikkim Himalaya.
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