ISSN No. 0974-6927

Volume 14, 2022

NBU Journal of Plant Sciences

समानो मन्त्रःसमितिःसमानी

Official journal of

Department of Botany

University of North Bengal

NBU JOURNAL OF PLANT SCIENCES

Volume 14, 2022

The NBU Journal of Plant Science serve as the official organ of the Department of Botany. The Journal publishes in English original research reports, review articles and Short communications ranging across all fields of Botany and Life sciences.

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Cover Photo

Top right corner: Alkali Spreading value of *Oryza sativa*, **Top middle**: In situ localization of Superoxide radicle in root of mung seedling, **Top left**: Habit and dissection of *Acampe ochracea* (Lindl.), **Bottom left**: Morphology of *R. stolonifer* sporangium under SEM, **Bottom middle**: Correlation network between isoflavonons and salt responsive genes, **Bottom right**: Habit and tubers of two local varieties of *Kochu.*

Technical Assistance: Ashis Sarkar & Raja Ghosh

From Editor's Desk

Over the course of human history, plants have received widespread and occasionally dominant aesthetic and intellectual attention. Research of plant systems can also instruct us on how to handle issues with agriculture, human health, and the environment. The NBU Journal of Plant Sciences (NBUJPS) is an international peer-reviewed journal that has been published by the Department of Botany, University of North Bengal, since 2007. It gives the platform for publishing top-notch research on a variety of topics in plant science, animal-plant interactions, environment and ecology, and the evolution of the living world.

The journal's editorial board publishes high-calibre articles in print and online (www.njps.nbu.ac.in) in one volume each year. The NBUJPS has officially registered for membership in CrossRef. All the articles will be assigned unique DOI numbers and will include other CrossRef services like similarity checking and reference linking. It gives me extreme pleasure to inform you that the NBUJPS publishes all the articles free of charge.

We are delighted to inform you that volume 14 has been published and will have 11 articles including review and research papers. This volume includes articles on nanotechnology, growth performance, characterization and ecology of various crops and weeds, phytoremediation of diseases and stresses in various crops, and microbes in extreme climates. These articles were contributed by numerous senior professors, scientists, and junior researchers from various institutions. The articles in this issue cover a wide spectrum of recent advancements in both basic and applied fields of plant science research, and they are all very important. Regarding contemporary breakthroughs and need-based research, this volume will greatly benefit all scientists and researchers working in various sectors of plant sciences.

Prof. (Dr.) Monoranjan Chowdhury
Head, Department of Botany

OBITUARY

Dr. Palash Mandal, Associate Professor

Dr. Palash Mandal (Ph.D.) was faculty of the Department of Botany, University of North Bengal, and was actively engaged in teaching and research in Plant Physiology, Pharmacognosy research, nanobiology including crop development and pharmacological activities, Bioprospectation and phytochemistry. Dr. Mandal passed away in the Siliguri on 21st July, 2022 after a short illness of pulmonary infection. He was 48 and is survived by his wife (Dr. Chandrani Chaudhuri) and his one daughter (Miss. Sneha Mandal) and son (Master. Rig Mandal). In his passing away, an excellent teacher of Plant Physiology and pharmacognosy has been lost. He will be remembered for his valuable contributions to botany of India. Dr. Mandal, born in 1974 in Kalyani of the district Nadia, and had obtained his B.Sc. (Hons.) degree in Botany in 1994. He was awarded with Gold Medal as he secured first position in M.Sc. in Botany from the University of Kalyani in 1996. He was awarded his Ph.D. degree from the University of North Bengal in 2013 under the supervision of Prof. P.K. Sircar & Prof. P. K. Basu. In 2001, he joined the department as Lecturer of Botany and successively held the positions of Assistant Professor (S-I), Assistant Professor (S-II), Associate Professor. He was successfully played his role in various administrative bodies like Executive council, PGBoS, Sports Board, Library Committee etc. of University of North Bengal. In connection with annual botanical excursions, conference and research he had visited different parts of India with the Post Graduate students and Ph.D students of Botany. Dr. Mandal became familiar and acquainted with the incredible knowledge of Plant physiology and Pharmacognosy in the country. His academic achievements and lifelong dedication to these areas of study earned him prestigious memberships in various scientific societies viz., Life membership in Indian Journal of Sericulture, Central Silk Board, Ministry of Textiles, GOI; Memberships in Society for Ethnopharmacology and East Himalayan Society for Spermatophyte Taxonomy.

He was successfully completed a total of 7 major and minor research projects. His completed research projects were as follows- 1. Evaluation of physico-chemical feasibility of manufacturing process for maintaining the antioxidant properties in different grades of tea of North Bengal (**UGC** [F. No. 34-536 / 2008 (SR), Dated 15.01.2009]); 2. The Profile study of peptides and antioxidants of Mulberry leaves: in relation to their potential in artificial diet rearing system of silkworm (UGC [F. No. 39 – 346 / 2010 (SR), Dated:

01.02.2011; 2011-2014); 3. Developmental stimulation of antioxidants in dark germinated *Trigonella foenum-graecum* L. by Nitric Oxide Donors and Scavengers (University of North Bengal [Ref. No. 773/R-13 dated 14.02.2013]); 4. Improving the economic Attributes of silk work rearing by mulberry leaves elicitation with Plant Growth Regulators (University of North Bengal [Ref. No. 114/R-2018(SF-15) dated 14.02.2013]); 5. Inventorization of Sacred Groves in (Uttar and Dakshin Dinajpur Districts of West Bengal (West Bengal Bio-Diversity Board [490 / 5K(Bio)-6/2017 dated 29/06/2017]); 6. Improving the productivity in sericulture by oligopeptides and plant growth regulator (PGR) based post-harvest elicitation of mulberry leaves (Department of Science & Technology, Govt. of West Bengal [263(Sanc.)/ST/P/S&T/1G-65/2017 Dated: 25.03.2018]); and 7. Molecular characterization and assessment of the efficacy of low molecular weight peptides isolated from mulberry leaves against flacherie disease of silkworm (Central Silk Board [No. CSB-31/2(BER-NP)/2017-18/RCS Dated 10.05.2019]).

Dr. Mandal has published more than 120 research articles in journals of national and international repute. He had also published 5 reference books entitled Electrophoretic Separation: Laboratory handbook (Palok Publishers), Pristine Treasure Trove: Exploring Sacred Groves of Uttar Dinajpur, West Bengal, India (LAP), A quick look into Chitosan and Chitosan nano-conjugates (LAP), Handbook on Bio-Staining Procedures: A Quick Practical Approach (Orange Book Publishers) and Basic Bio-Chemistry: A fundamental concept for the Beginners (HSRA Publications). He had supervised the research works of 7 students for their Ph.D. degree. He had participated in many national and international seminars, workshops and symposia including XIX IBC in Shenzhen, China. Dr. Mandal will remain alive within the hearts of his students, Research scholars and colleagues. New generation of researchers will continue to further advance our understanding through his recent works in the field of oligopeptides in plant physiological system, physiology of sprouts and upregulation of important metabolites through priming, pharmacognostic characterization and pharmacological evaluation, synthesis of nanoparticles using green technology and its application in economic field and enhancing the economic attributes of silkworm through elicitation of mulberry leaf quality.

The Department of Botany, University of North Bengal has always remembered him as being deeply saddened by his untimely demise because of his immeasurable and intolerable commitment to the improvement of department through his insightful teaching, research, and ideas. His abrupt passing is sincerely mourned by the students, researchers, plant enthusiasts, and entire fraternity of plant scientists, who also pray to God to grant his family and those close to him the strength they need to cope with this irreparable loss.

M. Chowdhury
University of North Bengal

Articles

NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 1-7

Review Article

Persepectives on Extremophilic Actinobacteria - A Review

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Abstract

Actinobacteria are considered as the most potential and biotechnologically viable prokaryotes because of their ability for the production of bioactive metabolites. They have immense biosynthetic prospect that remains unopposed without a competent organism from other microbial collections. But the prospect of finding highly potential actinobacteria from ambient habitats is reduced due to the wide exploitation for antibiotic production. So attention has been diverted to the unexploited extremophilic habitats such as marine sediments, mangroves, deserts, rocks, glaciers, etc. Extremophilic actinobacteria are competent producers of new secondary metabolites that show a wide range of antagonistic activities against bacteria, fungi, cancer and also exhibit insecticidal and enzyme inhibition. This review is an attempt to explore extremophilic actinobacteria that may form the source for the synthesis of novel drugs that could be used to combat resistant pathogens and also for xenobiotic degradation

Article info

Received **21 December 2022**
Revised **21 April 2023**
Accepted **27 April 2023**

Keywords: Actinobacteria; Extremophile; Secondary metabolite; Xenobiotics; Antibiotics.

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.001***

Introduction

Extremophiles are organisms competent enough to grow under extreme conditions that are particularly antagonistic to humans and to the majority of the known microorganisms as far as temperature, pH, and salinity parameters are concerned (Horikoshi et al, 2010). They have been isolated from habitats characterized by hydrostatic pressure, aridity, radiations, high temperatures, extreme pH values, high salt concentrations, and high solvent/metal concentrations, and it is well authenticated that these organisms are capable of thriving under extreme conditions better than any other organism living on Earth. Extremophiles have also been explored as far as the search for life on other planets is concerned (Stan-Lotter,2007). They are fascinating tools for the study of basic and applied science due to their unique structural and physiological features which help them to thrive in extremely selective environmental conditions. These properties are often attributed to specific biomolecules (DNA, lipids, enzymes, osmolites, etc.) that have been used as novel sources for biotechnological applications (Canganella and Wiegel, 2011). Actinobacteria which is a large phylum in the microbial community exhibits wide diversity in their habitat (Al-Shaibani et al, 2021). Many of the genera in this phylum have been isolated from extremophilic conditions. Generally, they are mostly aerobic, gram-positive to gram-variable with high G+C content, having mycelial or nonmycelial growth, and occupies diverse ecological niche (Amin et al,2020). Though many *Actinobacteria* produce a mycelium, which is a nonseptate and slenderer-like filamentous fungus, and many of them reproduce by sporulation (Chater and Chandra, 2006). However, the comparison to fungi is only superficial: like all bacteria, actinobacteria cells are thin with a chromosome that is organized in a prokaryotic nucleoid and a peptidoglycan cell wall; and the cells are susceptible to antibacterial agents (Smith, 2005).

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Actinobacteria mostly inhabit in soil, freshwater, and marine habitats playing an important role in the degradation of organic compounds, such as cellulose and chitin, thereby playing a vital part in biogeochemical cycles, replenishing the supply of nutrients in the soil, and aiding in humus formation (Anandan et al,2016). A phylogenetic study based on 16S rRNA classifies actinobacteria into six classes i.e., Acidimicrobia, Corniobacteria, Nitriliruptoria, Rubrobacteria, Thermoleophilia, and Actinobacteria (Sen et al. 2014). The class Actinobacteria contains 16 orders and the order Actinomycetales is now restricted to the members of the family *Actinomycetaceae*, and the other suborders that were previously part of this order are now designated to specific orders (Salam et al, 2020). This phylum is recognized as a producer of a wide variety of secondary metabolites with different activities including herbicides, antifungals, antitumor or immunosuppressant compounds, and anthelmintic agents (Manivasagan et al., 2014). However, the phylum has adapted to a wide range of ecological environments (Goodfellow and Williams, 1983) that are of adverse conditions such as acidic/alkaline pH, low or high temperatures, salinity, high radiation, low levels of available moisture, and nutrients (Zenova et al, 2011). This is possible due to their high metabolic and physiological flexibility in terms of several surviving strategies such as the production of enzymes, chaperones, varied nutrient adaptations, antibiosis, etc. (Mukhia et al,2021). Based on the different extremophile habitats they can be classified as alkaliphilic, thermophilic, acidophilic, radio-tolerant, halophilic, etc.

Thermophiles

Thermophilic actinobacteria flourish at relatively high temperatures ranging from 40 to 80°C (Shivlata and Satyanarayana,2015, Tortora et al., 2007). They are widespread, and commonly found in moldy hay (Corbaz et al., 1963), self-heating plant residues, cereal grains, sugar cane bagasse (Suihko et al., 2006), decaying vegetable materials, and compost heaps (Henssen and Schnepf, 1967). They can be strictly thermophilic and moderately thermophilic in nature. The former can grow in the temperature range between 37 and 65°C, but optimum proliferation takes place at 55–60°C. The moderately thermophilic actinobacteria thrive at 28–60°C and require 45–55°C for optimum growth (Jiang and Xu, 1993). Another group known as thermotolerant actinobacteria can survive at temperatures up to 50°C (Lengeler et al., 1999). Some examples of thermophilic actinobacteria are *Amycolatopsis ruanii and Amycolatopsis thermalba Thermopolyspora, Thermomonospora, Thermotunica, Thermocatellispora, Thermobispora, Acidothermus, Acidimicrobium, and Thermoleophilum.*

Acidophiles

Mostly, actinobacteria grow in soils with a neutral pH. They grow best at a pH between 6 and 9, with maximum growth around neutrality. However, a few strains of *Streptomyces* have been isolated from acidic soils (pH 3.5) (Kim et al, 2003). Other Acidophilic Actinobacteria, which are common in terrestrial habitats such as acidic forest and mine drainage soil, grow in the pH range from about 3.5 to 6.5, with optimum rates at pH 4.5 to 5.5 (Poomthongdee et al,2015). For instance, *Streptacidiphilus anmyonensis* sp. nov., *Streptacidiphilus rugosus* sp. nov., *Streptacidiphilus melanogenes* sp. nov. (Cho et al, 2008) grow well in acidic soil. Acidophilic and acid-tolerant actinobacteria have been studied as promising strains in biotechnology.

Xerophiles

The organisms which can grow in xeric or desert conditions are known as xerophiles. Desert soil is also considered an extreme terrestrial environment where certain species of actinobacteria, often use *Microcoleus, a cyanobacterium* as a source of food (Anandan et al,2016). The distribution of actinobacteria in various locations, such as sandy soil (Cario, Egypt; Falmouth, MA), black alkaline soil (Karnataka, India), sandy loam soil (Keffi Metropolis, Nigeria; Presque Isle, PA), alkaline desert soil (Wadi El Natrun, Egypt; Wadi Araba, Egypt), and subtropical desert soil (Thar, Rajasthan), where *Streptomyces sp*. were dominant followed by the other organisms, such as *Nocardia, Nocardiopsis*, and *Actinomycetes* (Cundell and Piechoski,2016) are reported. The novel isolate recovered from a desert soil sample collected in Beni-Abbes (southwest Algeria) and named Nonomuraea sp. (Badji et al,2007) *Streptomyces youssoufiensis* sp.nov., was isolated from a Moroccan phosphate mine by Hamdali et al, 2011.In 2019, Nafis et al reported the isolation of different actinobacterial genera (*Streptomyces, Nocardioides, Saccharomonospora, Actinomadura*, and *Prauserella* from Moroccan desert soil of Merzouga, Draasfar mining sites which exhibits plant growth promoting activities. Bioactive compounds have also been identified in actinobacterial isolates from the Algerian Saharan desert (Badji et al., 2006), Atacama Desert (Rateb et al., 2011), Egyptian desert (Koberl et al., 2011), Qinghai-Tibet Plateau (Ding et

al., 2013), and Thar desert (Thumar et al., 2010). Saccharothrix sp. PAL54A strain isolated from Saharan soil in Ghardaïa produced the known chloramphenicol (Aouiche et al,2012); therefore, it is the first production of this antibiotic by a Saccharothrix species. Currently, the focus has been diverted to extremophilic actinobacteria with the hope that these organisms would add a novel dimension to antimicrobial products research (Zitouni et al., 2004, Vijayakumar et al., 2012, Dhanasekaran et al., 2014).

Psychrophiles

Psychrophiles (cold-loving organisms) are the most plenteous organisms on earth in terms of biomass, diversity, and distribution (Margesin et al,2008). Actinobacteria phylum has been reported as one of the more abundant microbial groups in different Antarctic regions (Cary et al., 2010; Pearce et al., 2012). It has been observed that very old permafrost contains an increased amount of Actinobacteria (Willerslev et al., 2004). Antarctic actinobacteria isolates belonging to genus *Arthrobacter, Streptomyces,* and *Rhodococcus* exhibited antifungal activities (Santos et al,2020). The genus *Arthrobacter* was isolated from alpine permafrost in China (Bai et al., 2006). The psychrophilic and psychrotolerant actinobacteria of *Nocardiopsis* and *Streptomyces* were isolated from the water samples of the Polar Frontal region of the Southern Ocean (Sivasankar et al,2014). Psychrophiles are subjected to temperature fluctuations and frequent freeze-thaw events. This has led to the evolution of a number of adaptation mechanisms with regard to reproduction, metabolic activities, survival, and protection strategies in these organisms (Teufel et al,2018). The actinobacterial isolates that were adapted to growth at low temperatures and alkaline conditions, produce a wide range of extracellular enzymes such as proteases, amylases, and cellulases (Zhang et al, 2007). Culture-dependent and culture-independent molecular methods and the emerging fields of genome and proteome analyses will provide further new insights into a psychrophilic lifestyle (Margesina and Miteva, 2011).

Lithophiles

The actinobacterial community is known for its role in ecological succession as one of the pioneer communities. There is a growing interest in stone-dwelling actinobacteria which exhibit several adaptations to thrive in their habitat. The major family which has been isolated from stone niche is *Geodermatophilaceae*. *Geodermatophilaceae,* an actinobacterial family (Normand, 2006) which is endemic to the soil in the order *Geodermatophiliales* (Sen et al., 2014) that comprises three genera: *Geodermatophilus, Blastococcus,* and *Modestobacter* initially isolated from desert soils (Luedemann, 1968), seawater (Ahrens and Moll, 1970) and Antarctic regolith (Mevs et al., 2000), respectively. They grow despite unfavorable conditions associated with stone including limited sources of nutrients and water, high pH, and exposure to extreme variations in temperature, humidity, and irradiation. These stone-dwelling microbes are often resistant to extreme environments including exposure to desiccation, heavy metals, and UV & Gamma irradiation (Ding et al,2022). These early ambassadors of life may give new insights into the evolution of life.

Halophiles

Halophiles are present in the salt-rich environment such as deep marine sediments, mangroves, salt lakes, etc. The marine environment is an emerging source of novel actinobacteria and thus of new metabolites. *Rhodococcus marinonascene,* the first marine Actinomycete species to be characterized, supports the existence of marine Actinobacteria (Helmke and Weyland, 1984). Marine actinobacteria present in the extremely diverse environment produce different types of bioactive compounds compared with terrestrial ones (Ward and Bora, 2006, Makkar and Cross 1982). They had to adapt from extremely high pressure and anaerobic conditions at temperatures just below 0- 8 °C on the deep-sea floor to high acidic conditions at temperatures of over 8- 100°C near hydrothermal vents at the mid-ocean ridges (Bull et al,2005). Members of the genera *Dietzia, Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris,* and *Verrucosispora* are indigenously marine actinobacteria (Jenson et al, 2004). Currently, there has been a growing awareness of the potential value of marine water habitats as source of actinobacteria that produce useful metabolic products.

Another important source of a hypersaline environment is mangroves. Mangrove forests are the ecosystems prevalent in the tropics and subtropics; they make up over a quarter of the total coastline in the World (Saddhe et al,2016). They provide a habitat for different flora and fauna and also an abode of the microbial biome. The microbes in mangrove habitats not only produce primary and secondary metabolites but are also involved in soil organic matter decomposition and mineralization (Ghosh et al. 2011). In the anoxic mangrove

rhizosphere, Actinobacterial species such as *Streptomyces, Micromonospora*, and *Nocardioform* were found to be abundant (Tan and Cao,2009). Similarly, *Nocardia* isolated from mangrove soil produced new cytotoxic metabolites that strongly inhibited human cell lines, such as gastric adenocarcinoma (Schneider,2009). There is an ongoing interest in the isolation and characterization of actinobacteria and in to study of the enzymatic adaptations present in them.

Cave Dwellers

Caves are rarely studied and contain different mineral formations, permafrost and previously unknown organisms that have evolved in the micro-environment with more or less constant temperature, humidity, air composition, and other parameters over extended periods of time (Culver DC, Sket B,2000). These ecosystems are of great interest to microbiologists, due to the presence of microorganisms, which have been subjected to evolutions in stable conditions for a long duration (Grady F,2005). Actinobacteria are reported to be a dominant microbial population in several cave ecosystems (Groth and Saiz-Jimenez, 1999; Cheeptham et al., 2013; Tomczyk-Zak and Zielenkiewicz, 2016; Ghosh et al., 2017). The majority of the novel actinobacteria were isolated from cave soils including 6 novel genera, *Antricoccus, Beutenbergia, Knoellia, Lysinibacter Spelaeicoccus* and *Sphaerimonospora* (Rangseekaew and Pathom-Aree, 2019). The genus *Hoyosella* was recovered from the complex biofilm on the ceiling and wall of Altamira cave, Spain (Jurado et al, 2009). The extreme conditions within the caves are supposed to create stress for the inhabiting microorganisms at the genetic level, leading to the evolution of new species (Tiwari and Gupta, 2013). Therefore, caves are considered a prospective source for the isolation of novel actinobacterial taxa.

Conclusion and Future Perspectives

Actinobacteria which is a primitive and prominent phylum among prokaryotes are distributed in a wide range of ecological niches. They are encountered with different lifestyles such as plant commensals, nitrogen-fixing symbionts, as well as animal and plant pathogens. Thus, they constitute a significant proportion of the telluric microflora which is of extensive interest to the scientific community. The studies have proven that extremophilic actinobacteria are efficient producers of new secondary metabolites which show a wide range of biological activities including antibacterial, antifungal, anticancer, and insecticidal properties and enzyme production.

The pure culture and isolation of microbes from their natural environment posed a severe constraint in the recent past. The advent of 16S RNA analysis and metagenomic studies are helping us to remove this drawback. Furthermore, actinobacteria with its diverse portfolio is an ideal group of organisms to study xenobiotic metabolism. This field is gathering momentum because of the increased efflux of xenobiotics by anthropogenic activities.

Yet, actinobacterial research for the most part is rather recent and knowledge of many members is still elusive. This review is an attempt to give a comprehensive account of actinobacteria, especially from extremophilic habitats based on the knowledge available today.

Acknowledgments

AS acknowledges Biswa Bangla Genome Center, University of North Bengal. GS, IS acknowledge Bioinformatics Facility Center, University of North Bengal. All authors acknowledge Department of Botany, University of North Bengal.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 8-20

Research Article

Study of Major Isoflavones in Mungbean Seedlings with Special Emphasis on Its Enhanced Antioxidant Activity After Solid Matrix Priming with Selected Elicitors Including Nano-Chitosan Under Salinity Stress

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Abstract

For a long time, mung bean has been a well-liked crop. It is frequently used as a popular dish in the primarily cereal-based diets of Asian countries for its physiological functionalities, such as antioxidant, antitumor, and antidiabetic activities. Isoflavones present in legume-based foods have high antioxidant potential. These isoflavones are considered beneficial to human health and are linked to a reduced risk of cardiovascular disease, osteoporosis, and the prevention of certain types of cancer in humans, including breast, prostate, and colon cancer, as well as menopausal symptoms. On the other hand, nanotechnology is starting to look like an excellent method to boost food production and make farming less hazardous to the environment. Fascinatingly, the seed nano-priming method demonstrated promising results to mitigate the detrimental effects of different abiotic stress factors including salinity stress on crop plants and has thus, led to higher crop yields. The current study aimed to evaluate the effects of solid matrix priming (SMP) using nano-chitosan in mung bean sprouts under salinity stress related to the production of major mung bean isoflavones, which were detected through high-resolution liquid chromatography-mass spectrometry. When compared to unprimed seedlings exposed to salinity stress conditions, phytochemical quantification showed that SMP with nano-chitosan showed improved antioxidant activities as well as the highest total flavonoids and proline content. Under salinity stress, SMP with nano-chitosan significantly increased the biochemical anti-oxidative properties in germinated mung bean seeds, and also provided salt tolerance. As a familiar healthier choice, and because of the significance of mung bean sprouts for human health and the industry's rapid expansion, nutritional enrichment of this food has emerged as a significant field of study.
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Article info

Received **19 December 2022**
Revised **22 April 2023**
Accepted **28 April 2023**

Keywords: Mung bean sprouts; Isoflavones; Nano-chitosan; Salinity; Solid matrix priming

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.002***

Introduction

Legumes from the Fabaceae family are the primary source of isoflavones (Dixon and Sumner 2003) and mung bean [*Vigna radiata* (L.) R. Wilczek] has long been a popular leguminous crop that is frequently used as a popular dish in the predominantly cereal-based diets of Asian countries and accepted all over the world for its physiological functionalities, such as antitumor, antioxidant, and antidiabetic activities (Li et al. 2012; Yao et al. 2013). It is a well-balanced source of vitamins, minerals, fiber, protein, and bioactive compounds in significant amounts (Gan et al. 2017). According to studies, mung beans have physiological properties that include anti-obesity,

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anti-oxidation, and antibacterial effects (Yao et al. 2013). In many developing nations, mung beans offer a high-quality, all-natural plant protein source that has been utilized in place of meat and dairy products (Connolly et al. 2015; Du et al. 2018). Mung bean polysaccharide has been shown in studies to have antioxidant and immunoregulatory properties (Lai et al. 2010). Additionally, the mung bean's essential fatty acids can aid in the body's growth and development. Mung beans are a significant source of phytochemicals, such as phenols and flavonoids, which have health-promoting properties like antioxidants, anti-tumor, and radiation protection, in addition to their nutritional value (Randhir and Shetty 2007; Soucek et al. 2005). Nanotechnology in the form of seed nano-priming is an emerging field of study and intriguingly, the seed nano-priming strategy has been displayed to be effective against various abiotic stress factors, and it has also increased yields of crops (Abbasi Khalaki et al. 2021; Ye et al. 2020). Among the various seed priming techniques, solid matrix priming (SMP) is an efficient and less frequently used innovative method which is quite advantageous over liquid or osmotic priming (Sen & Mandal 2018). In SMP, wet seeds are combined with a solid substance and their moisture content is adjusted lower than that needed for seed sprouting (Harman & Taylor 1988). Seed priming, which involves applying nanoparticles to seeds to stimulate germination and subsequent plant growth by triggering the physiological functions of plants and giving resistance to various stresses, is a novel and cost-effective method. Priming seeds with nanoparticles stimulates electron transfer and boosts the capacity of the seeds' surfaces to react to substances found in the cells and tissues of the plant. By Seed nano-priming, the equilibrium between plant growth hormones and reactive oxygen molecules can be maintained and the biochemical processes can also be controlled (de Espirito Santo Pereira et al., 2021). Chandrasekaran et al. (2020) found that when nanoparticles are taken up by the seed coating, they might boost the generation of reactive oxygen species, work through various metabolic processes, raise the amount of active gibberellins, and get proteins out of storage. Additionally, the increased water intake by the seeds as a result of nanoparticles' effects can stress the seeds enough to trigger germination and boost the activity of several enzymes throughout phases I and II of the germination process (Joshi et al. 2018). Due to the enhanced activity of enzymes including superoxide dismutase, catalases, and guaiacol-peroxidase under stress circumstances, nanoparticles can operate to lower seed ROS levels, minimizing seed damage (Guha et al. 2018). Since seed sprouting is the first step in improving a crop, it could be used as a way to measure whether nanomaterials are beneficial or detrimental to the crop (Ahmed et al. 2019). Several genes, including those involved in the plant's tolerance to stress, have recently been shown to be triggered by seed nano-priming when sprouting is in progress (An et al. 2020; Ye et al. 2020). Plant-based foods containing isoflavones are thought to have high antioxidant potential and be good for human health. Isoflavones are one of four plant-based phenolic substances, along with stilbene, coumestan, and lignan, that fall under the category of phytoestrogens, which are estrogen-like compounds, structurally similar to 17-estradiol and can bind to estrogen's alpha and beta receptors (Cornwell et al. 2004; Sirotkin and Harrah 2004). The roles of the alpha and beta estrogen receptors vary. While beta estrogen receptors are in charge of cell apoptosis, alpha estrogen receptors are involved in cell proliferation (Rietjens et al. 2013). Due to their ability to block intracellular signaling pathways linked to NF-kappa B and immune responses, phytoestrogens have an impact on the immune system. Specific immune reactions and lymphocyte proliferation can be inhibited by genistein (Jefferson and Williams 2011). Additionally, isoflavones have the same antioxidant, anticancer, antimicrobial, and anti-inflammatory properties as other flavonoids (Conklin et al. 2007; Dhayakaran et al. 2015; Rodríguez-Roque et al. 2013; Chacko et al. 2007). Isoflavones may be able to scavenge free radicals, reduce low-density lipoprotein and DNA susceptibility to oxidative stress, and increase the activity and expression of antioxidant enzymes, according to the research on their antioxidant effects (Erba et al. 2012). In plants, phytoestrogens do not function as hormones, but as phytoalexins, synthesized and accumulated in plants during stress and microbe attacks. These active defense compounds have fungistatic, antibacterial, antiviral, and antioxidant properties (Dakora and Phillips 1996). They also prevent angiogenesis, thereby being important in the fight against malignant tumors (Bellou et al. 2012). In plants, isoflavonoids play many roles in plant-microbe interactions, including rhizobia-legume symbiosis and defense responses (Rípodas et al. 2013). In addition, they are also reported to be involved in abiotic stress responses. Their levels under stress depend on the

studied plant and stress type. Several studies have reported that UV-A and UV-B light have a positive influence on isoflavone accumulation (Liu et al. 2017), although some other stress reports are also not unambiguous. For example, in a study by Swigonska et al. (2014), after long and short-duration cold stress, osmotic stress, and combined cold and osmotic stress, the content of all the identified isoflavones such as daidzein, genistein, etc., increased in the roots of soybean seedlings. It is already reported that low-concentration salt stress could increase the total isoflavone content in chickpea sprouts (Gao et al. 2015). Similar results were also observed in tobacco and soybean plants with enhanced accumulation of the metabolites along with the beneficial impacts of isoflavones on plant salt tolerance (Jia et al. 2017).

In order to determine how solid matrix priming with nano-chitosan affects seedling vigor in mung beans subjected to salinity stress and how it affects the antioxidant potential of mung beans as well as the major mung bean isoflavones with health benefits for humans, the current study has been designed.

Materials and methods

Collection and sterilization of mung bean seeds

From the Pulse and Oilseed Research Station in Berhampur, West Bengal, India, a mung bean cultivar (SAMRAT) was procured. Previous research on salt stress sensitivity assessment by Sen et al. (2016) on five most popular mung bean cultivars of west Bengal viz, Samrat, Sonali, Panna, Sukumar, and Bireshwar, indicated that Samrat was the most salt tolerant cultivar. Hence, SAMRAT has been chosen for the current study. The seeds were surface sterilized for 3 to 5 minutes in 0.1% mercuric chloride ($HgCl_2$), followed by several rinses in sterile distilled water.

Preparation of nano-chitosan

The well-known ionic gelation method was used to prepare nano-chitosan in the laboratory using low molecular chitosan and sodium tripolyphosphate (STPP) as a cross-linking agent (Rajeshwari et al. 2016). Chitosan powder was dissolved in 100 ml of a 2% acetic acid solution, weighing about 0.2g. After 15 minutes of stirring, the chitosan solution was thoroughly combined. The obtained chitosan solution was stirred continuously with a magnetic stirrer as the STPP solution, which had been prepared in distilled water, was added drop by drop. The setup was continuously stirred until the emulsion's milky color appeared. Notably, STPP is a safer ingredient when compared to other chemical cross-linkers.

Characterization of nano-chitosan

Microstructural analysis of the morphology, shape, and size of the dried nano-chitosan sample was carried out using scanning electron microscopy (SEM) (Shende et al. 2014). Celite and the dried mixture of Celite-nano chitosan were both subjected to SEM. Zetasizer (ZETASIZER NANO ZS90 ZEN3690) was employed to investigate the size and distribution of the nano-chitosan particles using the Dynamic Light Scattering (DLS) method (Zaki et al. 2015). The structural characteristics of the particles were ascertained by Fourier Transform Infrared (FTIR) utilizing KBr pellets (Anusha and Fleming 2016).

Germination setup and sample preparation

In separate airtight zipper bags (10.5 cm X 7.8 cm) containing 1g of celite (used as a matrix) that were kept at a 10% water content using nano-chitosan, sterilized seeds were appended for seed priming. Additionally, one more set of seeds was used as a control (i.e., unprimed). So in the current experiment, two treatments viz., chitosan primed and nano-chitosan primed seeds were used together with an unprimed control, with three replicates of each treatment. After 24 hours of priming, the seeds were taken out of the matrix (celite), dried, and stored refrigerated overnight. The seeds were maintained for seven days under 4 dSm^{-1} salinity stress conditions while they germinated in the seed germinator (REMI) set to 25 ± 2°C. The saline solution's electrical conductivity was measured using a conductivity metre (dSm^{-1} = Deci Siemen per metre). After that, a sample of the seedlings was prepared for biochemical tests.

Determination of proline content

With a few minor modifications, the method outlined by Bates et al. (1973) was employed here to determine the free proline content in mung bean seedlings.

Determination of hydrogen peroxide (H_2O_2) content

According to the procedure outlined by Loreto and Velikova (2001), hydrogen peroxide accumulation was measured.

Determination of lipid peroxidation (malondialdehyde content)

Malondialdehyde contents were calculated using the Davenport et al. (2003) recommended technique to estimate the extent of lipid peroxidation. The following formula was used to determine the amount of MDA present:

MDA content (µmol/g) = [6.45 (A_{532} -A_{600}) – 0.56A_{450}] × V_t/W Where A_{600}, A_{532}, and A_{450} represent absorbance at 600, 532, and 450 nm and V_t = 0.0021 and W = 0.2 g.

Determination of enzymatic antioxidant activity

The mung bean samples (0.5 gm) were homogenized in a pre-cool mortar and pestle to assess the enzymatic antioxidant activity. The crushed sample was combined right away with 0.1 M ice-cold potassium phosphate buffer, which contains 0.5 mM EDTA and has a pH of 7.5 for catalase and superoxide dismutase and pH 6.8 for ascorbate peroxidase. The centrifuged supernatant after centrifugation at 15000 × g for 15 min at -10˚C from the crushed buffer sample from the centrifugation tube was used for the enzymatic assay.

Detection of superoxide dismutase (SOD) activity

The Esfandiari et al. (2007) method was used to estimate the activity of superoxide dismutase.

Detection of catalase (CAT) activity

The detection method prescribed by Aebi (1984) was followed for estimating *Catalase* activity with certain modifications.

Detection of ascorbate peroxidase (APX) activity

According to Chen and Asada's method (1989), ascorbate peroxidase activity was estimated. APX was expressed in terms of unit, where one unit was defined as the quantity of enzyme required to put away 1 µm of ascorbate min^{-1} mg $protein^{-1}$.

Determination of non-enzymatic antioxidant activity

Determination of ascorbic acid content

Detection processes described by Omaye et al. (1979) were followed for estimating ascorbic acid content. Sample extraction was done by homogenizing 0.5 gm leaf samples in 10% (w/v) TCA, followed by centrifugation at 10,000 × g for 20 min at 25˚C to collect the supernatant. Detection was carried out by reacting 0.5 ml supernatant with 2% 2, 4-dinitrophenyl hydrazine in 0.5 N sulphuric acid and 10% thiourea. The absorbance of the reaction mixture was recorded at 520 nm after 3 hrs of incubation at 37˚C.

Detection of flavonoid content

The content of flavonoids was determined at 510 nm following the protocol of Atanassova et al. (2011) by adding 300 µl 5% $NaNO_2$, 300 µl 10% $AlCl_3$, and 2 ml 1 (M) NaOH to the aqueous extract and by using quercetin as standard. An assessment of the bioactive potential of primed seedlings of mung bean was conducted in terms of antioxidant activity.

Quantitative profiling of free radical scavenging activities

The antioxidant activity of mung bean seedlings was evaluated in terms of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay and $ABTS^+$ (2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) scavenging assay compared with appropriate standards. For extraction, the first 1 gm fresh mung bean sample was homogenized in 10 ml methanol with mortar and pestle and thereafter, centrifuged at 10,000 × g for 10 min at 4˚C. The method prescribed by Sidduraju et al. (2002), was followed for DPPH scavenging activity. DPPH scavenging activity was estimated at 517 nm by mixing 0.2 ml methanolic extract with 2 ml DPPH against ascorbic acid as standard. DPPH radicals scavenging activity was calculated as percentage inhibition according to the following equation: DPPH scavenging activity (%) = (A_0-A_1)/ A_0 × 100 Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The final result of antioxidant activity (DPPH scavenging activity) was expressed as IC25 (µg/mL), which represented the sample concentration which represented the extract concentrations scavenging 25% of DPPH radicals (Nishaa et al. 2012). ABTS radical scavenging activity was estimated following the method of Sidduraju et al. (2002), against butyl-hydroxytoluene (BHT) as standard. ABTS antioxidant activity was estimated using butyl-hydroxytoluene (BHT) as standard. ABTS free radical scavenging activity was calculated as percentage inhibition according to the following equation: ABTS scavenging activity (%) = (A_0-A_1)/ A_0 × 100 Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The final result of antioxidant activity (ABTS radical scavenging activity) was expressed as IC50 (µg/mL), which represented the extract

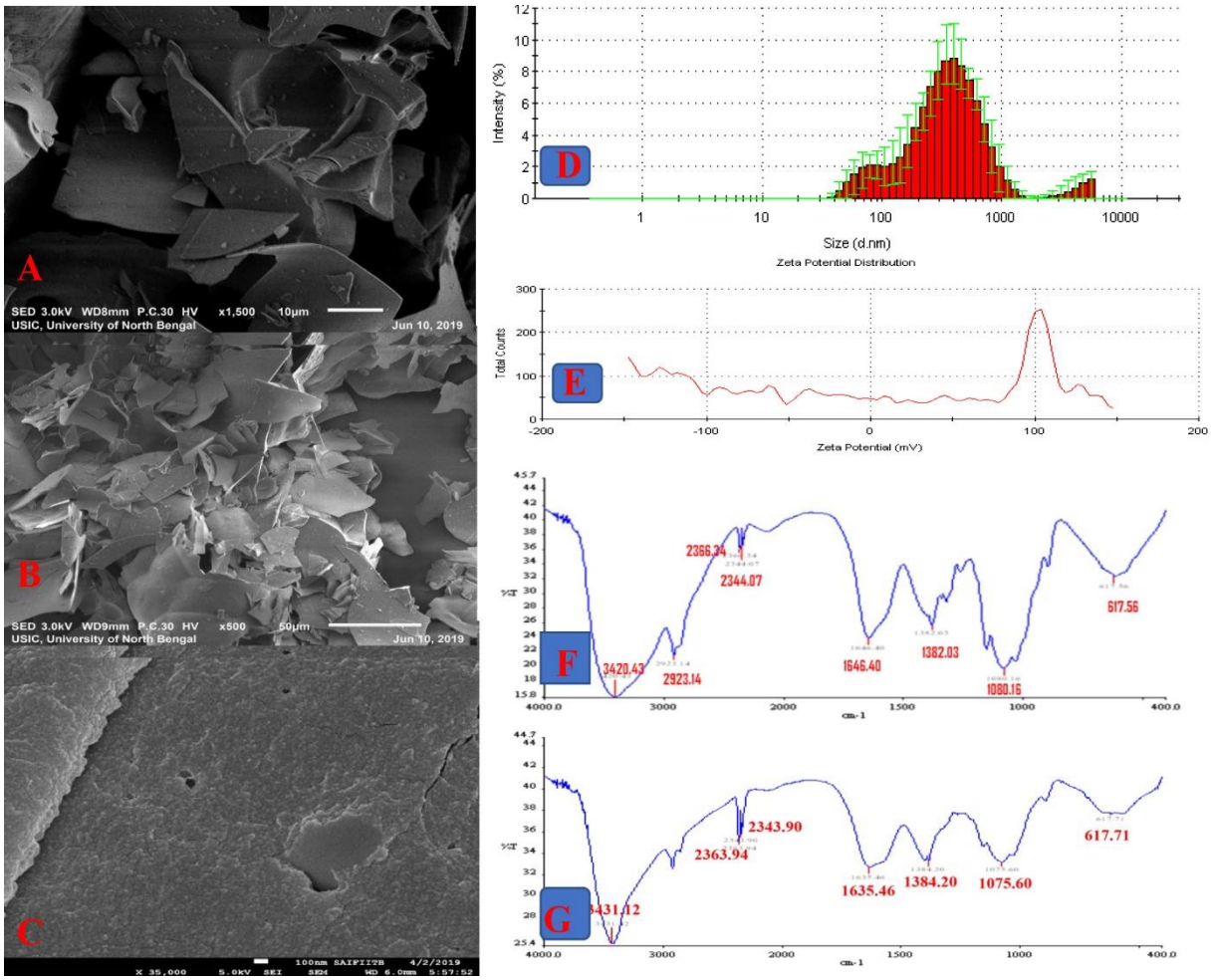

Fig 1. Characterization of nano-chitosan: (A) SEM of Celite, (B) SEM of Celite-nano-chitosan mixture, (C) SEM of nano-chitosan, (D) DLS of nano-chitosan, (E) Zeta potential of nano-chitosan, (F) FTIR spectra of chitosan and (G) FTIR spectra of nano-chitosan.

concentrations scavenging 50% of ABTS free radicals (Nishaa et al. 2012).

Principal component analysis (PCA) and Heat map

PCA and a heat map were created in this study using ClustVis Software and the heatmap R package (version 0.7.7). One of the methods in the pcaMethods R package is used to calculate the principal components.

High-resolution liquid chromatography-mass spectrometry (HR-LCMS) analysis:

HR-LCMS of untreated and treated mung bean seedlings were conducted from SAIF-IIT Bombay.

Results and discussion

Scanning electron microscopy (SEM)

Fig. 1A and **Fig.** 1B show scanning electron microscopy (SEM) of Celite and Celite-nano-chitosan mixture respectively. It is evident from the SEM image that celite particles are ordinarily very loosely arranged to have wide gaps between them while the SEM image of the Celite-nano-chitosan mixture shows a more compact arrangement. On the otherhand, **Fig.** 1C shows an SEM image of nano-chitosan, which highlights the amorphous nature of the nano-chitosan. It is a more or less uniform structure in contrast to chitosan powder which shows a non-porous, plain, and smooth structure in SEM as reported by Sudha et al (2014).

Dynamic light scattering (DLS) and Zeta potential

Numerous analytical tools with practical applications represent the distinctive modifications in nanoparticles that occur throughout their formation from the chitosan polymer. DLS optically quantifies the motion of the suspended particles as well as the size distribution of chitosan nanoparticles (Dubin 1967). The intensity of scattered light changes over time as a result of the constant, random Brownian motion of the chitosan nanoparticles in this particular dispersion. The poly-dispersity index (PDI) of the chitosan nanoparticles was determined to be 0.465 and the early correlation coefficient decay curve to be in the spectrum of 250 nm (**Fig.** 1D). In terms of size distribution, the chitosan-TPP binary electrolyte system under some circumstances consisted of prepared TPP cross-linked nano-chitosan. Chitosan nanoparticles were determined to have a zeta potential of +101 mV (**Fig.** 1E). The stability was greater with increased surface charge.

Fourier transform infrared (FTIR) spectroscopy

Fig. 1F and 1G represent the FTIR spectra of chitosan and nano-chitosan respectively. The intermolecular interaction of nano-chitosan is revealed by FTIR analysis. N-H, C-H, and O-H single bonds are responsible for the distinctive peak that is seen in the range of 4,000 to 2,500 in the spectrum of nano-chitosan. On the other hand, the recognisable peak in the nano-chitosan spectrum that appears between 2,500 and 2,000 represents triple bond absorption. In the spectrum of nano-chitosan, the distinctive peak can be seen around 2,000 and 1,500 and is caused by absorption from double bonds such C=O, C=N, and C=C. The strong and wide peak appearing around the 3420 cm^{-1} area is attributed to hydrogen-bonded O-H stretching vibration in the chitosan spectra. The FTIR spectra of nano-chitosan exhibited a shift of the tip of the 3420 cm^{-1} to 3431 cm^{-1} which becomes wider with higher relative intensity in the FTIR of chitosan. This reflects the enhancement of hydrogen bonding. Moreover, at 1646 cm^{-1} the peaks for N-H bending vibration of amine I and at 1382 cm^{-1} the amide II carbonyl stretch shifted to 1635 cm^{-1} and 1384 cm^{-1} respectively. The presence of a P-O peak, which is clear from the FTIR measurements, also supported the relationship between the phosphoric and ammonium ions.

Biochemical analysis

Isoflavone content and derivation of the mung bean Isoflavonoids (KEGG pathway):

The group of secondary plant metabolites known as isoflavones is distinct and results from the phenylpropanoid pathway. They are mostly produced by Papilionaceae family members (Wang and Murphy 1994). **Fig. 2** displays the chromatograms obtained through quantitative LC-MS analysis of three major isoflavones namely (A) Biochanin-A, (B) Formononetin, and (C) Genistein which were found in mung bean sprouts.

The KEGG pathway of the flavonoid biosynthesis pathway could be used to explain how mung bean isoflavonoids are produced. As per the KEGG pathway, Genistein is derived from Naringenine via 2-Hydroxy-2, 3-dihydrogenistein ($C_{15}H_{12}O_6$). 2, 7, 4'-Trihydroxyisoflavanone acts as the substrate for the reaction that produces genistein, which is catalyzed by the enzyme 2-hydroxyisoflavanone dehydratase [EC: 4.2.1.105]. The enzyme isoflavone 4'-O-methyltransferase [EC: 2.1.1.212 2.1.1.46] then directly transforms genistein into Biochanin-A ($C_{16}H_{12}O_5$) (**Fig. 3**).

S-Adenosyl-L-methionine + Genistein <=> S-Adenosyl-L-homocysteine + Biochanin A

Genistein may also form Genistein 7-O-beta-D-glucoside ($C_{21}H_{20}O_{10}$), Prunetin ($C_{16}H_{12}O_5$), or 2'-Hydroxygenistein ($C_{15}H_{10}O_6$) by the enzyme isoflavone 7-O-glucosyltransferase [EC: 2.4.1.170], isoflavone-7-O-methyltransferase [EC: 2.1.1.150], and 4'-methoxy isoflavone 2'-hydroxylase [EC: 1.14.14.90 1.14.14.89] respectively. Flavanone liquiritigenin (7,4′-dihydroxyflavanone) is the precursor of Daidzein, Formononetin, and Glycitein; on the other hand, the precursor of Genistein and Biochanin-A is Naringenin (5,7,4′-dihydroxyflavanone) (Ko, 2014). By the presence of the enzyme 2-hydroxyisoflavanone synthase [EC: 1.14.14.87] Liquiritigenin or 4',7-Dihydroxyflavanone ($C_{15}H_{12}O_4$) gives rise to 2,7,4'-Trihydroxyisoflavanone ($C_{15}H_{12}O_5$) which in turn is converted into Daidzein ($C_{15}H_{10}O_4$) by the enzyme 2-hydroxyisoflavanone dehydratase [EC: 4.2.1.105] and converts Daidzein into Formononetin ($C_{16}H_{12}O_4$) by the activity of the enzyme isoflavone 4'-O-methyltransferase [EC:2.1.1.212 2.1.1.46] (**Fig. 4**).

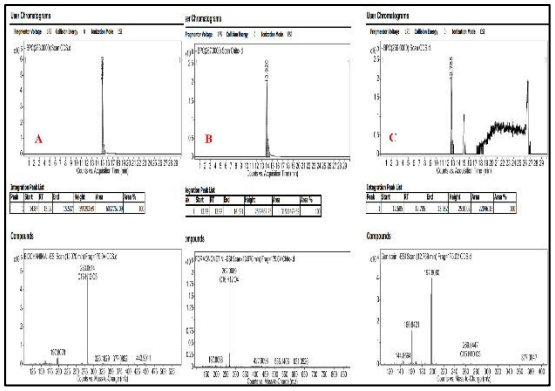

Fig. 2 HR-LCMS of three major isoflavones namely (A) Biochanin-A, (B) Formononetin, and (C) Genistein found in mung bean sprouts.

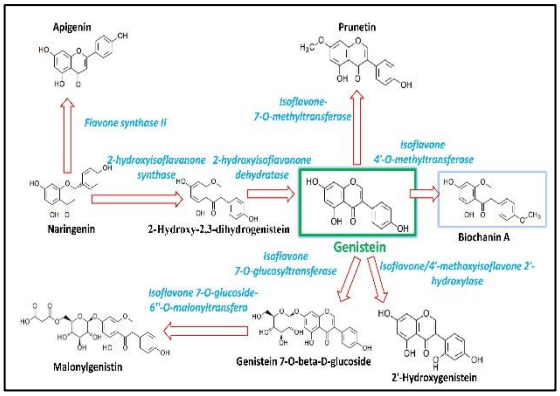

Fig. 3 Derivation and glycosylation of Genistein and Biochanin-A through the KEGG pathway

Antioxidant activity and oxidative stress management by SMP with chitosan and nano-chitosan

As compared to untreated plants, SMP with nano-chitosan significantly reduced the amount of H_2O_2 in the study. Similar patterns were visible in MDA content. The levels of MDA and H_2O_2 were higher in untreated plants. However, compared to untreated (control) plants, primed plants were found to have higher flavonoid, ascorbic acid, and proline contents, which may indicate that oxidative damage caused by ROS has reduced the integrity and stability of the plasma membrane. In general, plants exposed to nano-chitosan showed an increase in antioxidant activities like catalase (EC: 1.11.1.6) and ascorbate peroxidase (EC: 1.11.1.11). Similar to this, treated plants had higher PPO and peroxidase (EC: 1.11.1.7) activities than untreated plants did. In contrast to plants treated with nano-chitosan, chitosan-treated plants had slightly higher values. When compared to untreated plants grown under salinity stress, SOD values in treated plants showed a significant decline. SOD is a major O_2^- scavenger that catalyzes O_2^- to H_2O_2 and O_2 (Apel and Hirt 2004). After that, POD, CAT, or ascorbate peroxidase can be used to remove the hazardous H_2O_2 (Foyer and Noctor 2005; Kusvuran et al. 2020). Ascorbic acid was used as a benchmark for the ABTS radical scavenging activity. Previous publications have described the use of ascorbic acid, a common water-soluble antioxidant found in foods, as a standard for the ABTS assay (Tang et al. 2010). Similar outcomes in the DPPH assay were attained. High levels of H_2O_2 in mung bean plants are a sign of excessive salt-induced ROS production and are caused by membrane lipid peroxidation as a result of salt-induced oxidative damage. These findings in various plants grown under salt stress are supported by numerous reports (Shabala and Munns 2012). The SMP-induced improvement over salinity stress in terms of its alleviation is demonstrated in the current study by lower levels of H_2O_2 and MDA measured in treated mung bean plants.

Proline levels were found to be higher in plants treated with nano-chitosan, which also scavenges ROS and stabilizes biomolecules and bio-membranes. In order to restore their water balance, stressed plants collect osmolytes in the interior of their cells. The increase in Proline content in response to salt stress suggests that mung bean plants can adapt to osmotic stress brought on by salt. The SMP treatment with nano chitosan improved this capacity for dealing with salt stress even more, as shown by the continued rise in Proline content. Proline is widely known for its semi-protective

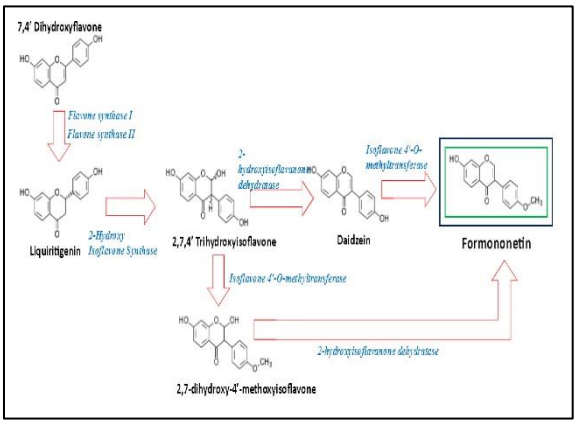

Fig. 4 Biosynthesis of Formononetin from flavones through the KEGG pathway

property and hydroxyl radical scavenging activity, which stabilizes bio-membranes and biomolecules both structurally and functionally (Ahmad et al. 2010). By defending the photosynthetic apparatus, proline also improves photosynthesis. As a result, it helps plants survive and adapt to salt stress by acting as an energy storage material. The osmoregulation, ROS scavenging, and stabilization of membranes as well as macromolecules like protein and DNA improved after SMP treatment with nano chitosan under salt stress in mung bean plants. The current study found that SMP with nano chitosan increased the level of proline in mung bean plants under salt stress, which is consistent with a previous report on pro levels in cadmium-stressed mung bean (Nahar et al. 2016). The current study's findings thus suggest that SMP containing nano-chitosan can reduce oxidative damage and control the solidity of the plasma membrane system under salt stress.

PCA biplots & Heat map analysis

PCA, a multivariate statistical technique, is frequently used to keep as much information as possible while reducing the dimensionality, or the number of variables, of a large number of correlated variables. Thus, the multivariate data set undergoes a linear transformation into a set of uncorrelated variables that are organized in descendant form by the variance explained. The PCA biplot in this study illustrates the relationships between variables including MDA, proline content, ascorbic acid content, and all of the enzymological assays carried out (POD, APX, CAT, PPO, and SOD) (**Fig.** 5).

The first principal component (PC1) shapes 84.15% and thus most of the studied attributes confined around it. Except ABTS, DPPH and flavonoid all other attributes are on the negative axis of PC1. Antioxidant enzymatic activity correlated positively

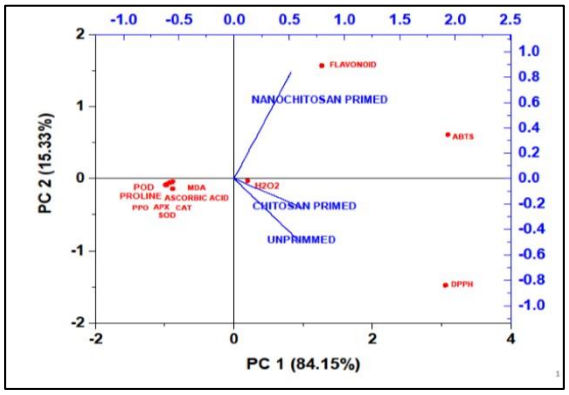

Fig. 5 PCA biplot of different treatments and biochemical attributes studied showing antioxidant properties.

with stress indicators, suggesting a defensive response to stress induction. However, when looking at stress markers in terms of IC50, antioxidant activity was found to have a negative correlation. Thus, PCA clustering clearly shows that mung bean seeds primed with chitosan and nano-chitosan are prospective enough to enhance seed germination by removing pre-germination stress through activating defensive secondary metabolites.

On the other hand, the heat map is a data matrix that uses a color gradient to show the values in the cells. In the present investigation, the heat map (**Fig.** 6) between the chosen parameters and characteristics of mung bean seedlings in the current investigation provides a clear overview of the highest and lowest values in the matrix. Evidently, nano-chitosan primed seedlings clearly showed enhanced activities of APX, PPO, POD, CAT and SOD. Proline content and MDA content were also significantly improved after nano-chitosan priming in comparison to the unprimed and chitosan treated ones.

Cytoscape analysis

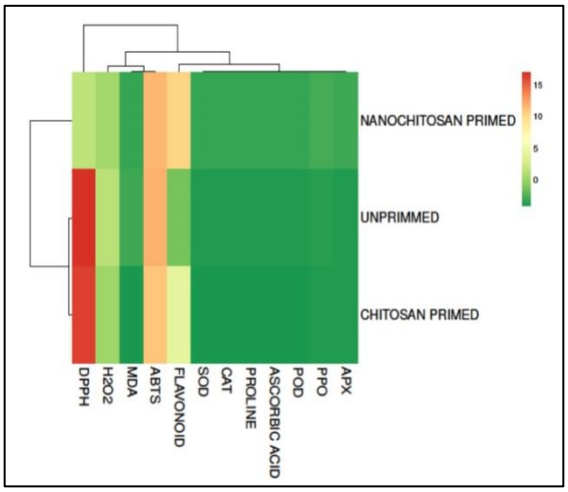

Fig. 6 Heat map between the selected parameters and attributes of mung bean seedlings grown under salt stress conditions.

The relationship between the chosen isoflavones and the salt stress genes is shown in **Fig.** 7. It is discovered that certain genes, including SOS1, SOS2, SOS3, CBL1, CBL10, NHX1, AKT1, and VPS34, play a role in the plant body's response to salt stress (pathway ID GO: 0009651). Furthermore, potassium ion homeostasis is regulated by the genes SOS3, CBL3, and NHX1 (pathway ID GO: 0055075). Ion transporters, such as the Na^+/H^+

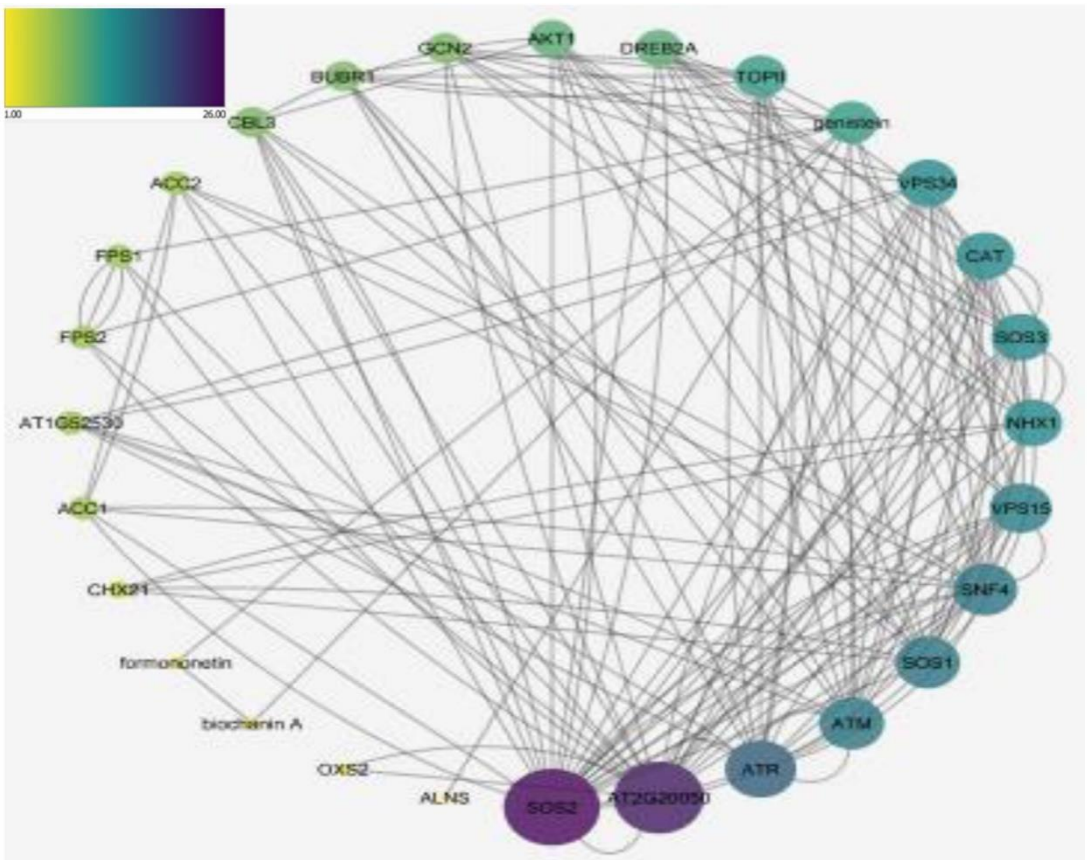

Fig. 7 The correlation between the selected isoflavones and the salt stress genes is represented through Cytoscape analysis.

antiporter SALT OVERLY SENSITIVE 1 (SOS1) and the K^+ rectifier ARABIDOPSIS K^+ TRANSPORTER 1 (AKT1), correctly maintain a high K^+/Na^+ ratio in the cytoplasm, a phenomenon essential for salt tolerance (Chen et al. 2005; Feng et al. 2015). The electrochemical gradient created by transmembrane proton pumps, such as plasma membrane H^+-ATPase, vacuolar H^+-ATPase (V-ATPase), and vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase), energizes these secondary transporters (Chen et al. 2010; Yang et al. 2010; Yuan et al. 2016). It is noteworthy that the plasma membrane H^+-ATPase is a critical site susceptible to various stresses, including salt stress, cold stress, heavy metal stress, and stress from active transport of the stressor across the plasma membrane (Martz et al. 2006; Shi et al. 2008; Janicka-Russak et al. 2012). In Arabidopsis thaliana, fatty acid biosynthesis (KEGG pathway ID 00061), fatty acid metabolism (KEGG pathway ID 01212), and pyruvate metabolism (KEGG pathway ID 00620) are all regulated by the genes ACC1, CAC1, and BCCP2. The genes BCCP2, CAC1, KIN11, ACC1, ACC2, and VPS34 are also involved in the cellular lipid metabolic process (pathway ID GO: 0044255) in Arabidopsis thaliana. Surprisingly, acetyl-CoA is also a factor in histone acetylation, giving peroxisomal FA oxidation control over nuclear epigenetic modification that may have an impact on a variety of cellular processes (Wang et al. 2019).

Conclusions

In the present investigation, the seeds were only treated with nano-chitosan for 24 hours, but the triggering impact was seen for a few days. In this case, it could be said that the NPs are taken up by the surface of the seeds and slowly released over the course of a week to have their effect. This study looked at how SMP with nano-chitosan affected the antioxidant activities of mung bean sprouts, especially the isoflavones found in mung beans, and their relationships to genes for salinity tolerance. When used as a priming agent in SMP, nano-chitosan not only improved the ability of mung bean seedlings to withstand salinity but also enhanced

their antioxidant capacities in comparison to untreated seedlings, which is linked to the metabolic shifting of important isoflavones with potential therapeutic applications. The results of this study suggest that SMP with nano-chitosan may be a useful technique for producing functional foods with high metabolic contents and isoflavones, which would enhance the nutritional value and health benefits of mung bean sprouts.

Acknowledgement

The valuable contribution of Mr. Kushankur Sarkar to accomplish the Cytoscape study is sincerely acknowledged.

Conflicts of interest

There are no actual or potential conflicts of interest to declare.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 21-24

Short Communication

New Report on *Fusarium equiseti* Causing Yellow Leaf Disease of *Brassica juncea* (L.) Czern from Karandighi, Uttar Dinajpur, West Bengal, India

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Abstract

Yellow leaf disease in B-9 variety of yellow mustard [*Brassica juncea* (L.) Czern] was noticed in the area of Khowaspur, Karandighi, Uttar Dinajpur District, West Bengal. Irregularly shaped dull yellow regions along the leaf margin and even in the midrib of leaf were observed. *Fusarium equiseti* (Corda) Sacc. (ON783721.1) was isolated and identified as causal potent fungal isolate causing yellows in leaves of *Brassica juncea*.

Keywords: *Fusarium equiseti*, yellow leaf disease, *Brassica juncea*

DOI: *https://doi.org/10.55734/nbujps.2022.v14i01.003*

Article info

Received **12 October 2022**
Revised **08 April 2023**
Accepted **03 May 2023**

Introduction

Yellows symptoms in leaves of *Brassica juncea* was noticed during January and February 2022 form the area of Khowaspur (Lat 25.772671^0; Long 88.032848^0), Karandighi, Uttar Dinajpur District, West Bengal (Figs. 1 & 2). In January, the temperature varied between 13.3-24.7 °C and 16.0-28.7 °C in February. In January, the average humidity was 53%, while in February humidity was recorded as 45%. The initial signs of the disease were irregularly shaped dull yellow regions along the leaf margins. These areas then spread to the leaf midrib and turned tan to brown (Fig. 1C). Lesions could be sparse and dispersed throughout the leaves and densely packed over vast areas of leaves. After surface sterilizing the diseased leaf sample with 0.1% $HgCl_2$ for 2 min, followed by ethanol for 2 min, the leaf sample (1–1.5 cm) was chopped into small pieces and placed on PDA (Potato Dextrose Agar) medium. An antibiotic Monocef-O 100 (each 5 ml of the reconstituted suspension contains cefpodoxime proxetil IP equivalent to cefpodoxime-100 mg) was added in PDA medium to prevent any bacterial interference. On potato dextrose agar, a cefpodoxime proxetil IP equivalent to cefpodoxime-100 mg) was added in PDA medium to prevent any bacterial interference. On potato dextrose agar, a fungus was isolated from the diseased leaf. On the basis of morphology of the fungal mycelia, various shapes and sizes of conidia by compound and scanning electron microscopy, the fungus was supposed to be species of *Fusarium* (Fig. 3). For molecular identification, first step was DNA isolation followed by fragment of 18S rRNA gene that was amplified by ITS1 and ITS4 primers- ITS1-TCC GTA GGT GAA CCT GC GG and ITS4- TCC TCC GCT TAT TGA TAT GC. A single discrete PCR amplicon band of 1500 bp was observed when carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 18S rRNA gene was generated from forward and reverse sequence data using aligner software and finally the 18S rRNA gene sequence was used to carry out BLAST with the "nr" database of NCBI GenBank database. *Fusarium equiseti* (ON783721) was identified and submitted to NCBI GenBank (**Fig. 4**).

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Fig. 1: Sample collection site (A &B); Disease symptoms (C) in leaf of B9 variety of yellow mustard (*Brassica juncea*)

Fig. 2: Earth map and GPS location of the sample collection site from where diseased leaves of *Brassica juncea* were collected (A-C)

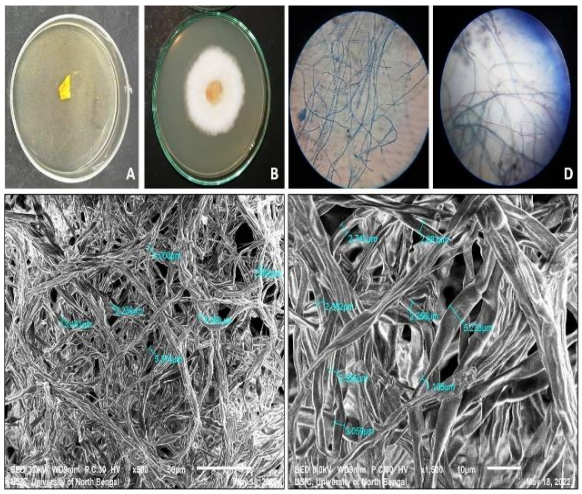

Fig. 3: Isolation on PDA medium (A); 7 days old growing isolate (B); Microscopic view of mycelia of

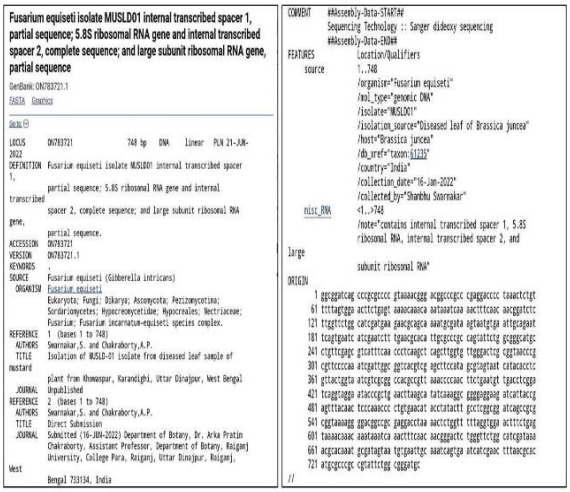

Fig. 4: Molecular identification of the isolate with GenBank accession number- ON783721

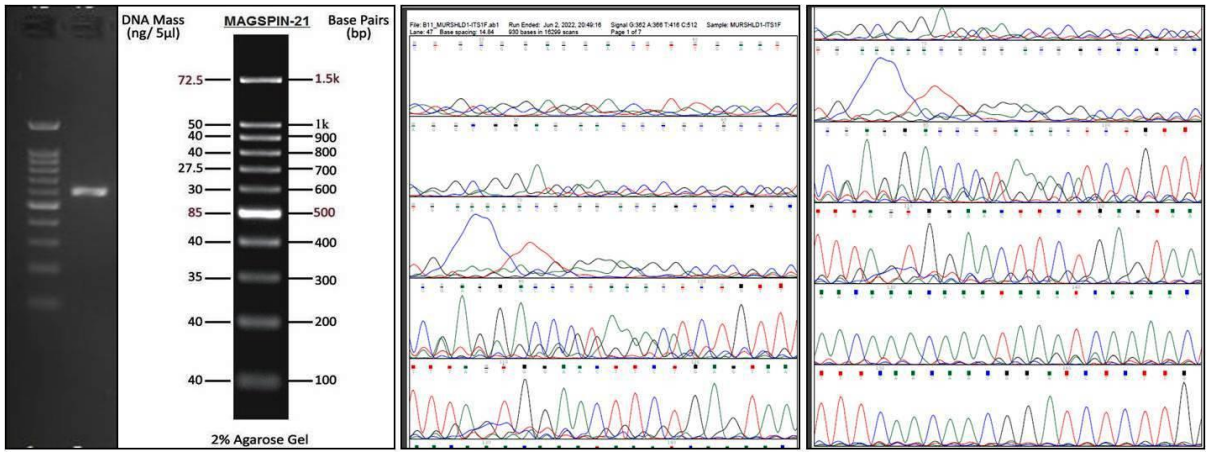

Fig. 5: Chromatogram of sequence of the fungus

resolved on agarose gel and forward and reverse DNA sequencing reaction of PCR amplicon was

The Neighbour Joining (NJ) method [Saitou and Nei (1987), Tamura et al. (2004, 2021)] was used to do phylogenetic analysis of *Fusarium equiseti* (ON783721.1) with other ex types of *Fusarium*. *Fusarium equiseti* (ON783721.1) was found to be closely related with *Fusarium equiseti* isolate MLS029 (OM203485.1) (Figs. 5 & 6). Healthy mustard plant [*B. juncea* (L.) Czern] was re-inoculated with foliar spray of spore suspension of *F. equiseti* (ON783721.1) and similar leaf symptoms were appeared. To confirm the attachment of fungal mycelia to the leaf surface, scanning electron microscopy of the leaf surface was performed and the existence of fungal mycelia was confirmed. After Koch's Postulate, the isolated fungal morphology

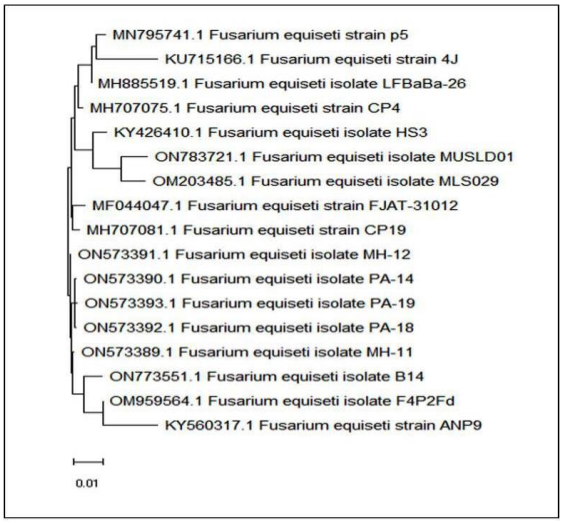

Fig. 6: Phylogenetic analysis of *Fusarium equiseti* (ON783721) was performed with other ex types of *Fusarium* by Neighbour Joining (NJ) method

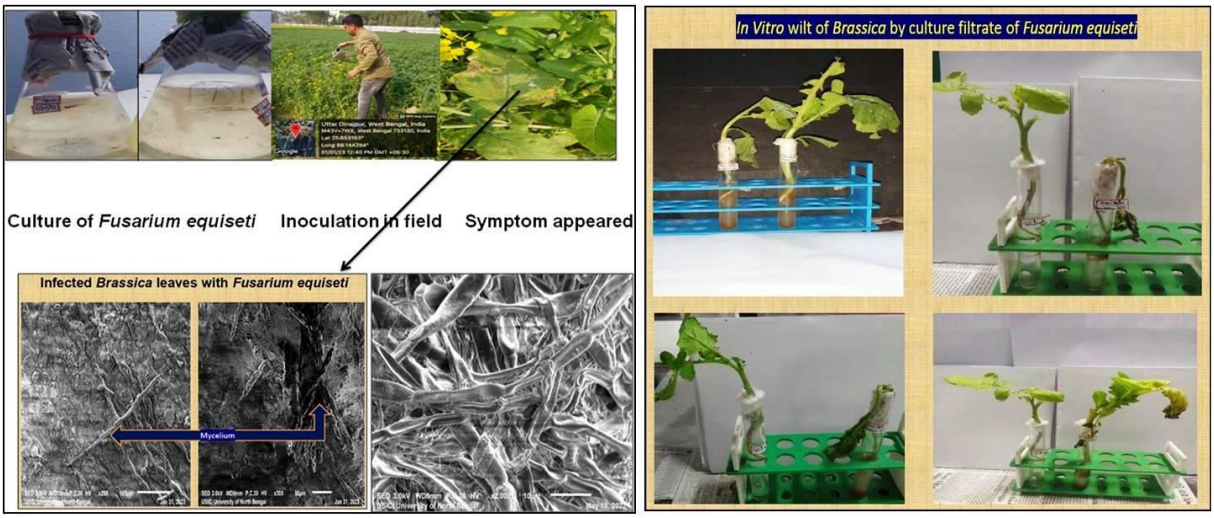

Fig. 7: Koch's postulate in field condition; establishment of leaf disease by *Fusarium equiseti* (ON783721) and *in vitro* wilt disease by *F. equiseti*.

was rechecked through scanning electron microscopy. *F. equiseti* (ON783721.1) was able to cause wilt of the plant when the plant was dipped into the spore suspension of the fungus *in vitro* condition (Fig. 7). This case report is regarded as new one on *Fusarium equiseti* (ON783721) causing yellow leaf disease of *Brassica juncea* from Karandighi, Uttar Dinajpur, West Bengal. Similar findings were found on first report of *Fusarium equiseti* causing wilt and seedling death in other crop plants in support of our new finding (Mishra et al. 2021; Khan et al. 2021; Astudillo-Calderón et al. 2019; Rajput et al. 2020; Aldakil et al. 2019).

Conflict of Interests

The authors declare no conflict of interests among them.

Acknowledgements

Authors take this opportunity to express earnest gratefulness to M/s Edison Life Science, Kolkata for molecular identification of the isolate and the Director of USIC-Scanning Electron Microscopy Unit, University of North Bengal for taking the scanning electron microscopic photographs.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp.25-31

Research Article

Characterization of Some Cultivated Rice (*Oryza sativa* L.) Based on Phenotypic, Physicochemical and Cooking Properties

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Abstract

Evaluation of rice genetic diversity is an important step for character specific varietal development program. The present study characterizes 15 rice germplasm of Indian accessions on the basis of agro morphological, physicochemical and cooking parameters. The cultivars showed high degree of variations on their traits. Mean plant height of 88.96 cm, flag leaf length (26.48 cm), flag leaf width (1.17 cm), grain per panicle (130), panicle length (20.97 cm), days to 50% flowering (112.06 days), kernel length breadth ratio (2.65), cooked kernel length breadth ratio (3.25), linear elongation ratio (1.65). Out of 15 rice cultivars, only Sada nunia and Das nunia are aromatic rice and Sada nunia also contain awn. Plant height showed significant positive correlation with PnL and DF and significant negative correlation with GB. GL positively significantly correlates with FLL, GrWt with GL and GB, DF with MT. The cluster analysis grouped the 15 cultivars into 4 clusters with 37.5 dissimilarity coefficient. The high variability with promising traits among the cultivars expected to be significance for future rice breeding programmes.

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Keywords: Agro-morphology, Genetic diversity, Germplasm, Cultivars, Variability, Cluster analysis

DOI: *https://doi.org/10.55734/nbujps.2022.v14i01.004*

Article info

Received **19 December 2022**
Revised **21 April 2023**
Accepted **18 May 2023**

Introduction

Rice (*Oryza sativa* L.) is one of the leading food crops of the world that feeds as a staple food nearly one half of the world's population (Singh et al., 2005). Rice is a cereal crop and belongs to the family Poaceae. Rice grain contains 7% protein, 12% water and 75 to 80% starch (Oko et al., 2012; Hossain et al., 2015). India is the major rice growing country and produces about 20% of all world rice. In India, out of total cropped land rice is cultivated in 44.7% area and produces 70.3% of the total food grain (DAC&FW 2018).

Rice is grown in more than 100 countries across all habitable continents. In South East Asian countries, India traditionally rich in the diversity of cultivated rice also in the wild progenitors of cultivated rice (Singh et al., 2001). Genetic diversity maybe provides as an insurance against crop failure (Subba Rao et al., 2001). Landraces contain many valuable traits such as production, quality, biotic and abiotic stress tolerance etc. Assessment of genetic diversity is extremely important in rice breeding for selection and conservation of different landraces for their further utilization in crop improvement programmes (Patra, 2000). The successful crop improvement programme is extremely dependent on the efficient exploitation of the genetic variability within germplasm and selection of the genotypes which contains quality contributing and desirable yield traits (Acquaah, 2012).

Hence, characterization of rice cultivars and their identification are important for the genetic improvement, seed production programs and release. Thus, assessment of these varieties will further donate towards creating a genetic database and will help in planning breeding programmes in that region.

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Materials and methods

Plant material

The experimental material consisted of 15 cultivated rices. The genotypes were grown in the pots as well as field at Experimental Rice Field, Plant Genetics & Molecular Breeding Laboratory, Department of Botany, University of North Bengal, West Bengal, India.

Agro-morphological traits

The data were collected on 10 randomly selected plants from each germplasm. Agro-morphological traits were measured according to DUS (Distinctiveness, Uniformity and Stability) test protocol (PPV&FR Acts 2001, Govt. of India) for characterizing the diverse genotypes. The 21 days old seedlings of each genotype were transplanted with spacing 20 cm × 20 cm in randomized complete block design (RCBD) with 3 replications. Observations like Plant height (PH), flag leaf length (FLL), flag leaf breadth (FLB), panicle length (PnL), grain per panicle (Gr/Pn), days to 50% flowering (DF), maturity date (MT), Tillering (Till), 1000 grain weight (GrWt), grain length (GL), and grain breadth (GB) were recorded.

Grain physico-chemical and cooking parameters

Based on Alkali Spreading Value (ASV), Gelatinization Temperature was measured. For Alkali Spreading Value, six kernels were taken and removed their aleurone layer manually with the help of blade. The polish kernels were kept in petriplate containing freshly prepared 1.7% KOH solution and covered them with lid. After 10 mins., lids were opened and smelt, graded them for aroma by sensory evaluation protocol by Sood and Siddiq (1978). After 23 hours of incubation at room temperature, data was recorded visually based on the appearance and disintegration of kernels and scored them according to Little et al. (1958). A low ASV score indicates to a high GT. Conversely, a high ASV score indicates a low GT.

Elongation ratio was measured according to Oko et al. (2012). The linear elongation ratio was calculated by the average length of cooked kernel divided by the average length of uncooked kernel and breadth wise elongation ratio was calculated by average breadth of cooked kernel divided by the average breadth of uncooked kernel. Cooked kernel length breadth ratio was calculated by length of cooked rice divided by breadth of cooked rice.

Statistical Analysis.

The experiment was conducted with three replications under laboratory condition. The experimental data were recorded in Microsoft Excel. The descriptive statistics were done in Microsoft Excel 2007. Pearson correlation coefficients (r) were calculated by using IBM SPSS 23 statistical software. Pair Group Method with Arithmetic Mean (UPGMA) hierarchical clustering in Past4.03 software based on dissimilarity matrix for phenotypic diversity among the rice cultivars.

Results and Discussion

Variability of morphological traits

Significant genetic variation was seen in the germplasm for the agro-morphological traits. Among the 15 germplasm, the traits under study showed wide range of variations (Table 1). Plant height (PH) ranges from 67.11 cm to 128.00 cm with a mean of 88.96 cm and 17.22 CV%. Flag leaf length (FLL) ranges from 19.09 cm to 36.01 cm with a mean of 26.48 cm (%CV = 19.29) and flag leaf breadth ranges from 0.61 cm to 1.59 cm with a mean of 1.17 cm (%CV=20.76). Panicle length ranges from 15.36 cm to 25.59 cm with a mean of 20.97 (%CV=11.94). Grain per panicle ranges from 85 to 176.33 with a mean of 130 (%CV=18.56) Grain length and breadth ranges from 7.28 mm and 2.04mm to 9.40 mm and 3.08 mm with a mean of 8.22 mm and 2.52 mm (%CV=7.99 and 11.78). Grain weight ranges from 16.40 g to 25.64 g with a mean of 20.85 (%CV=14.61). Tillering ranges from 3 to 7 with a mean of 4.13 (%CV=1.15). Days to 50% flowering and maturity time ranges from 98 days and 133 days to 128 days and 158 days with a mean of 112.06 days and 144.33 days (%CV= 7.39 and 5.05). Awns recorded mean of 2.33mm (%CV=313.61) respectively.

Physico-chemical and cooking parameter analysis

It was observed that rice grain quality was showed a huge genetic variability. Out of 15 rice cultivars only Sada nunia and Das nunia are aromatic and rest are non-aromatic. Alkali spreading value (ASV) was showed a wide range of genetic variability among the cultivars. Ranjana cultivar showed lowest Alkali spreading value (ASV)(1), so gelatinization temperature (GT) is highest (6) and Sada nunia showed highest ASV (6) and lowest GT (1) (**Fig. 1.**) (**Table 2**).

Table. 1 Agro-morphological variation of rice cultivars

Traits	Range	Minimum	Maximum	Mean	SEM (±)	Std. Deviation	CV (%)
PH	60.89	67.11	128.00	88.96	3.95	15.32	17.22
FLL	16.92	19.09	36.01	26.48	1.31	5.11	19.29
FLB	0.98	0.61	1.59	1.17	0.06	0.24	20.76
PnL	10.23	15.36	25.59	20.97	0.64	2.50	11.94
Gr/Pn	91.33	85.00	176.33	130.00	6.23	24.13	18.56
GL	2.12	7.28	9.40	8.22	0.16	0.65	7.99
GB	1.04	2.04	3.08	2.52	0.07	0.29	11.78
GrWt	9.24	16.40	25.64	20.85	0.78	3.04	14.61
Till	4.00	3.00	7.00	4.13	0.29	1.15	27.89
DF	30.00	98.00	128.00	112.06	2.13	8.28	7.39
MT	25.00	133.00	158.00	144.33	1.88	7.29	5.05
Awn	28.30	0.00	28.30	2.33	1.90	7.38	316.61

Plant height (PH cm), Flag leaf length (FLL cm), Flag leag breadth (FLB cm), Panicle length (PnL cm), Grain per panicle (Gr/Pn), Grain length (GL mm), Grain breadth (GB mm), Grain weight (GrWt g), Tillering (Till), Days to 50% flowering (DF days) and Maturity time (MT days), Awn (mm).

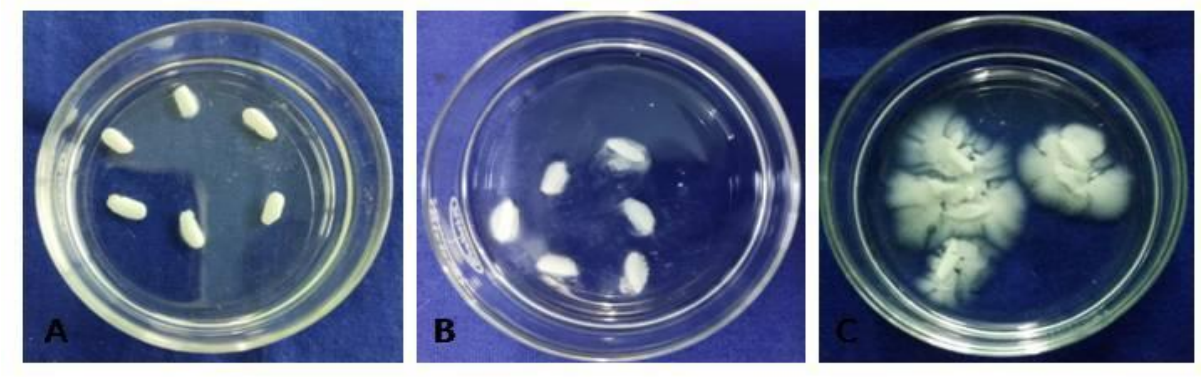

Fig. 1 Representative photo of ASV and GT experiment. Figure showing: A- Low ASV (1-2), B- Medium ASV (3-5), C- High ASV (6-7)

Table. 2 Physicochemical properties (ASV, GT, and Aroma) in rice cultivars.

Cultivar name	Aroma	ASV	GT
Mala	0	2	7
Das nunia	3	5	3
Swarno	0	2	7
China	0	2	7
IR-28	0	2	7
Sada nunia	3	6	1
Suruchi	0	3	5
Banni	0	4	3
Paras	0	3	5
Pioneer	0	3	5
Yamuna	0	3	5
Pratik	0	6	1
Nironjana	0	6	1
Ranjana	0	1	7
Paijam	0	6	1

Kernel length (KL) and Kernel breadth (KB) varied from 5.18 mm to 6.94 mm and 1.86 mm to 2.65 mm with a mean of 5.94 mm (CV% = 9.36) and 2.22 mm (CV%=8.88). Kernel length –kernel breadth ratio (KL/KB) is very important attribute of rice grain and it varied from 2.07 to 3.30 with a mean of 2.65 (CV%=14.91). Cooked kernel length (CKL) and breadth (CKB) varied from 8.00 mm to 11.16 mm and 2.40 mm to 3.80 mm with a mean of 9.78 mm (CV%=12.23) and 3.06 mm (CV%=11.27). Cooked kernel length-breath ratio (CKL/CKB) varied from 2.15 to 4.47 with a mean of 3.25 (CV%=20.78). Linear elongation ratio (LER) varied from 1.22 to 1.94 with a mean of 1.65 (CV%= 11.83) and breadth wise elongation ratio (BER) varied from 1.17 to 1.68 with a mean of 1.38 (CV%=10.67) (**Table. 3**).

Table. 3 Cooking properties of rice

Traits	Range	Minimum	Maximum	Mean	SEM (±)	Std. Deviation	CV (%)
KL	1.76	5.18	6.94	5.94	0.14	0.55	9.36
KB	0.79	1.86	2.65	2.22	0.05	0.19	8.88
KL/KB	1.23	2.07	3.30	2.65	0.10	0.39	14.91
CKL	3.26	8.00	11.26	9.78	0.30	1.19	12.23
CKB	1.40	2.40	3.80	3.06	0.08	0.34	11.27
CKL/CKB	2.32	2.15	4.47	3.25	0.17	0.67	20.78
LER	0.72	1.22	1.94	1.65	0.05	0.19	11.83
BER	0.51	1.17	1.68	1.38	0.03	0.14	10.67

Kernel length (KL mm), Kernel breadth (KB mm), Kernel length breadth ratio (KL/KB), Cooked kernel length (CKL mm), Cooked kernel breadth (CKB mm), Cooked kennel length breadth ratio (CKL/CKB), Linear elongation ratio (LER), Breadth wise elongation ratio (BER).

Correlation among the Traits

Pearson's correlation (r) measured the potency of the association between two characters. In the present study, the correlations among the characters showed significant positive relations among the traits (**Table. 4**). Plant height showed (PH) positive correlation with PnL, DF, MT, Awn and significant negative correlation with GB. Flag leaf length (FLL) showed positive correlation with GL. Grain weight (GrWt) positively correlates with FLB, GL and GB. Days to 50% flowering (DF) showed significant positive correlation with MT.

Table. 4 Correlation among agromorphological traits of 15 cultivars

	PH	FLL	FLB	PnL	Gr/Pn	GL	GB	GrWt	Till	DF	MT	Awn
PH	1											
FLL	.199	1										
FLB	-.155	.200	1									
PnL	.584*	.132	.055	1								
Gr/Pn	-.310	-.061	.183	-.145	1							
GL	.103	.565*	.408	.312	-.034	1						
GB	-.664**	-.109	.440	-.430	.173	.062	1					
GrWt	-.352	.111	.657**	-.136	.247	.563*	.606*	1				
Till	-.122	-.209	.090	.355	.129	-.118	.158	.143	1			
DF	.650**	.047	-.288	.192	-.207	.096	-.413	-.216	-.202	1		
MT	.562*	.277	-.334	.046	-.191	.188	-.362	-.279	-.370	.901**	1	
Awn	.453	.106	-.402	.503	-.125	.130	-.353	-.289	.265	.354	.368	1

*****Correlation is significant at the 0.05 level (2-tailed). ******Correlation is significant at the 0.01 level (2-tailed).

Plant height (PH cm), Flag leaf length (FLL cm), Flag leag breadth (FLB cm), Panicle length (PnL cm), Grain per panicle (Gr/Pn), Grain length (GL mm), Grain breadth (GB mm), Grain weight (GrWt g), Tillering (Till), Days to 50% flowering (DF days) and Maturity time (MT days), Awn (mm).

Diversity Analysis by Clustering

Based on 15 agro-morphological and physicochemical traits, a dendrogram was constructed by UPGMA hierarchical clustering methods and grouped the 15 rice cultivars into four clusters (**Fig. 2**) on the basis of average linkage and Euclidean distance. Cluster I consist Sada nunia and Das nunia. Cluster II consists five cultivars, Paijam Nironjana, Mala, Paras, Banni. Cluster III consist only Ranjana. Cluster IV consists seven cultivars namely Yamuna, Pioneer, Suruchi, Pratik, IR-28, China and Swarno.

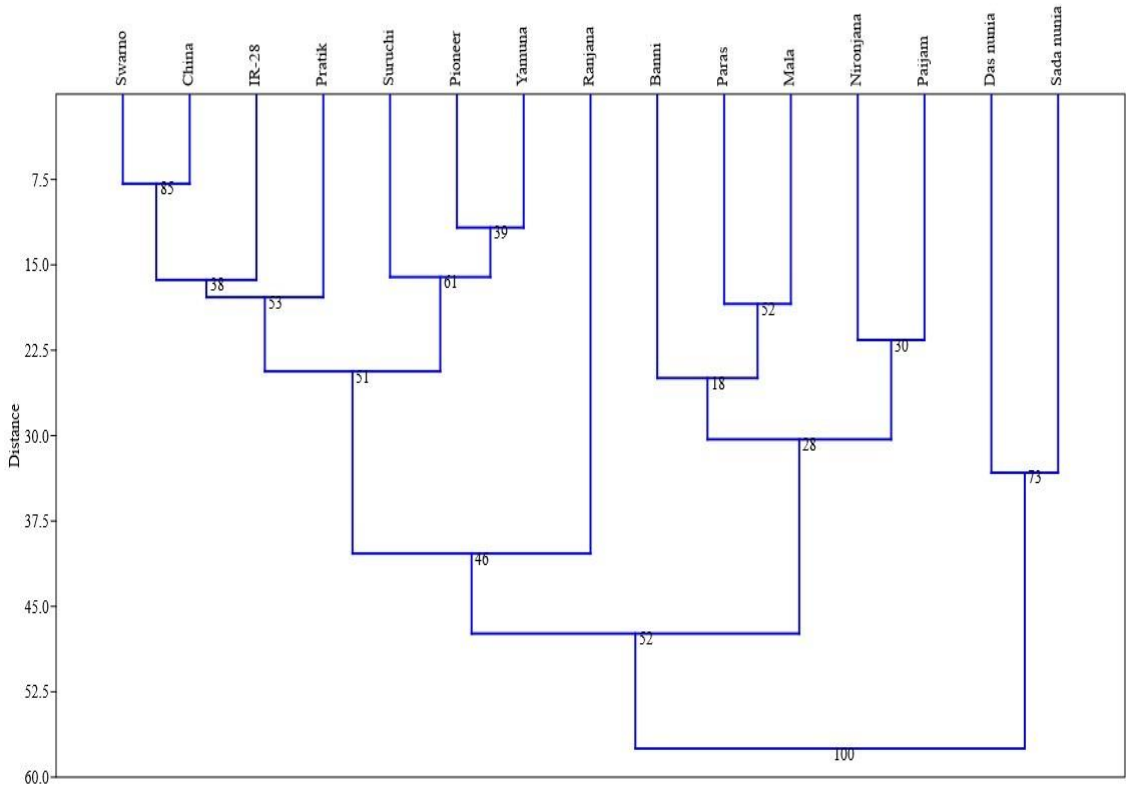

Fig. 2 Grouping of 15 rice cultivars based on unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering method.

Discussion

The characterization of germplasms on the basis of agro-morphological traits is fundamental for plant breeding programmes which provide the basic information. (Lin.,1991). The genetic analysis of quantitative characters is one of the main prerequisites for planning a plant breeding programme (Khatun et al., 2015). Among the 15 rice cultivars, all the traits show significant variations. Different phenotypic variations have been reported by many rice researchers in different rice accessions (Ullah et al., 2011; Pandey et al., 2011). The coefficient of variation (CV%) of phenotypic traits ranges widely (5.4% to 23.45%) and small variation on qualitative traits.

Gelatinization temperature (GT) is a temperature when rice starch started to bulge and lose its crystallinity in an irreversible manner. According to Surek 2002, gelatinization temperature is a physicochemical trait of the starch. Rice starch usually gelatinizes at 65 - 85 °C temperatures (Bakshi and Singh, 2019). During cooking, high gelatinization temperatures shows softening of rice. Grain size and shape mostly determine the purchaser acceptance and market value of rice, while cooking quality is controlled by the properties of starch. During cooking some rice varieties expand more in size than others. Length-wise elongation without a corresponding expansion in girth is considered a greatly desirable grain quality trait (Sood and Sadiq.,

1979). Cooked grain length is governed by genetic as well as environmental factors (Şişman, 2016).

Correlations assist the breeder to understand the interrelated characters in the time of selection for genetic improvement (Chakravorty et al., 2013). Plant height showed a significant positive correlation with PnL, supported by (Roy et al., 2014; and Saha et al., 2019). The negative correlation of the plant height with flag leaf area was also reported (Saha et al., 2019). The significant positive correlation of grain yield with 100-grain weight in the present study was supported by (Efendi et al., 2015; Ibrahim et al., 2019). Thus, the altering of these characters would have a positive impact on yield improvement. Days to maturity showed significant positive correlation with plant height reported in this study was supported by Krishna Naik et al. (2005). Grains per panicle showed negative correlation with plant height supported by Chakraborty and Chakraborty (2010).

The UPGMA dendrogram generally clustered the rice cultivars in to four major groups at 37.5 dissimilarity coefficient, which suggest a high level of phenotypic diversity in the rice genotypes.

Genotypes of distant clusters showed wide spectrum of variability in the segregation and to perform maximum heterosis in crossing (Hosan et al., 2010). Variability assessment of rice genotypes on the basis of cluster analysis is reported by several workers (Ghalain (2006), Hien et al. (2007), Naik et al. (2006), Sarawgi and Bhisne (2007) and Ratho (1984). Ratho (1984) reported that the pattern of clustering did not go after the geographical origin of a variety. For higher variability in breeding, parent plant selection on the basis of wider inter-cluster distances was recommended by Mishra et al. (2003), Chaturvedi and Mourya (2005). The present study provides strategy for selection of parents on the basis of agro-morphological traits with special reference to yield and quality characters for further improvement.

Conclusion

All the valuable rice cultivars possess huge genetic variability, which can be utilized in broadening the genetic base or varietal improvement program in future. Characterization of genotypes is the basic fundamental tool for breeding which helps to select the right parent plant. This study provides the basic information for rice variety improvement as well as breeding programs.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 32-34

Research Article

Preliminary Observation on The Ecological Amplitude of *Hypoestes phyllostachya* Baker in Darjeeling and Kalimpong Himalayas

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Abstract

Hypoestes phyllostachya Baker ("Polka dot Plant") is an exotic species that is quite problematic weed of Madagascar origin in Darjeeling and Kalimpong regions of Eastern Himalayan Vegetation. Present study was carried out in 26 different localities covering a vertical distribution from an altitude of 90 msl (Teesta Bazar) to 2,478 msl (Senchal Lake) and horizontal distribution from Rimbik (27.1182° N, 88.1084° E) to Bindu (27.0977° N, 88.8713° E), which revealed its very high invasive potential and ecological amplitude. Its presence in agricultural land together with forest and open land has been a matter of concern as it is creating a threat to the local floras. It was strongly felt that its control measure has to be implemented immediately in order to restore the ecological balance in these local areas.

Article info

Received **13 December 2022**
Revised **07 May 2023**
Accepted **19 May 2023**

Keywords: Weed; Invasive species; Ecological amplitude; Control measure; Horizontal and vertical distribution.

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.005***

Introduction

Hypoestes phyllostachya Baker commonly known as "Polka dot plant" belongs to Acanthaceae family is a plant of Madagascar origin. It is thought to be a foreign element and has very high invasive nature (Annon, 2016a, Moktan, 2017). Therefore, it is considered as one of the most problematic weeds (Annon, 2016b), particularly in Eastern Sub-Himalayan region. The increasing population of such exotic species in local region has been a great threat to the local floristic environment which may cause enormous loss of genetic diversity and ultimately species extinction.

While working on the plant resources of Mahananda Wildlife Sanctuary located in the Darjeeling district of West Bengal, Kumar et al. (2009) added new record of *H. phyllostachya* from West Bengal. Similarly, this species was also reported from the State of Kerala as an addition to the Flora of India (Remadevi and Binojkumar, 2001). This species was originally described from Madagascar and is also distributed in North America (Kumar et al., 2009). However, its frequent presence in many places of Darjeeling and Kalimpong districts of West Bengal prompted us to conduct the survey to find out the ecological amplitude as well as its distribution pattern in these two districts of West Bengal.

Materials and Methods

Regular surveys were conducted covering different seasons and various regions (table 1) of Darjeeling and Kalimpong hills. Relevant samples were collected, and herbarium sheets were prepared (Paul et al, 2020). Collected samples were identified using available literatures and also by comparing the herbarium sheets at the Herbarium of Department of Botany, Kalimpong College. Local farmers and tea garden workers were also asked regarding the invasion problems and other related issues of *Hypoestes phyllostachya* in their localities.

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Table. 1 Places where surveys were carried out.

Sl. No.	District	Localities	Altitude (M)	Latitude/Longitude	Date of collection
1	Darjeeling	Sungma T.E.	1710	26.938088° N & 88.178831° E	26/10/2019
2		Bunkulung	622	26.7957° N & 88.3118° E	11/10/2020
3		Nagri T.E.	1215	26.9121° N & 88.2096° E	11/10/2020
4		Dhajea T.E.	1050	26.9240° N & 88.2193° E	11/10/2020
5		Mirik (near lake)	1495	26.8908° N & 88.1825° E	08/08/2021
6		Sukhia Pokhri	2194	26.9984° N & 88.1669° E	30/10/2019
7		Rangbhang (near bridge)	1160	26.9002° N & 88.1975° E	08/08/2021
8		Ghoom (near railway station)	2558	27.0008° N & 88.2437° E	30/10/2019
9		Maneybhanjyang bazar	1928	26.9879° N & 88.1209° E	30/10/2019
10		Rimbik bazaar	2286	27.1182° N & 88.1084° E	10/11/2021
11		Deer Park Kurseong	1482	26°53'10.3"N & 88°17'27.5"E	22/10/2019
12		Sonada	1873	26.9599° N & 88.2680° E	22/10/2019
13		Lebong	1809	27.0615° N & 88.2765° E	22/11/2021
14		Tiger Hill (near Senchal Lake)	2487	26.9932° N & 88.2657° E	22/11/2021
15		Lapchu bazaar	1500	27.0600° N & 88.3654° E	25/08/2021
16		Teesta bazaar	90	27.0662° N & 88.4246° E	25/08/2021
17		Darjeeling bazar (Mal Road)	2080	27.0428° N & 88.2652° E	22/11/2021
18	Kalimpong	Kalimpong College Campus	1259	27.0595° N & 88.4669° E	10/03/2018
19		Lava	2345	27.0863° N & 88.6615° E	10/08/2018
20		Loleygoan	1670	27.02071°N & 88.565018°E.	10/08/2018
21		Gorubathan	417	26.9542° N & 88.6952° E	18/14/2018
22		Div Chowk	1275	27°02'33.6"N & 88°27'38.9"E	10/03/2019
23		Dr. Gram's Homes campus		27.0836° N & 88.4922° E	10/10/2021
24		Seed Farm, RRS, UBKV Campus	990	27.0717° N & 88.4799° E	12/10/2021
25		4th Mile	1100	27°4'0.0048" N & 88° 28' 0.0012" E	12/10/2021
26		Bindu (Jaldhaka)	609	27.0977° N & 88.8713° E	26/09/2020

Results and Discussion

Hypoestes phyllostachya is a 30 cm (12 in) tall, evergreen herb with heavily pink or white spotted leaves. It produces small, many solitary pink/purple flowers and fruits are many-seeded dehiscent capsule. The species are found with high seed germination rate and mostly growing on margins of forests, agricultural and wild areas. Total 26 locations were surveyed covering the altitudinal range from 90 msl (Teesta Bazar) to 2,487 msl (Senchal Lake, Senchal Wild Life Sanctuary) and 27.0977° N, 88.8713° E (Bindu, Jaldhaka) to 27.1182° N, 88.1084° E (Rimbik, Darjeeling) latitude-longitude gradients. In almost all the regions, *Hypoestes phyllostachya* were found growing luxuriantly. Its presence was abundant in the open spaces, mainly east and south east facing hill slopes. Forest floor and forest margins were also frequently covered by this species. Its presence was also noted in tea garden areas in some places (e.g., Sungma T.E., Nagri T.E., Dhajea T.E., etc). In those areas, it was not only seen in the open areas, but was also frequently present in between tea bushes.

Some farmers from the surveyed areas were of opinion that the polka dot plant has been a

problematic weed in agricultural field since recent past, as it has been invading new places in a very fast rate. Although the fields growing annual crops like maize, ginger, cauliflower, and other seasonal vegetables have been found to be less invaded by this weed, however, perennial crops like large cardamom growing field and mandarin orange orchard have been affected significantly. The reason may be that the manual weeding is done at regular interval in the annual crop fields, while in cardamom fields such activity is done once in a year before the harvest and only once or twice such manual weeding is done in mandarin orchards.

According to the tea garden labours, the growth of *Hypoestes phyllostachya* is very difficult to control. Before 2005, some tea gardens practised the usage of inorganic herbicide and weedicide to control the over growth of tea garden weeds. However, the chemicals were found to be least effective to control this species. Comparatively, the manual weeding (uprooting) was found to be effective in controlling the weed's growth.

In this present study, the distribution pattern of *H. phyllostachya* was found to be quite interesting, as its ecological amplitude has been found to be wide. The vertical distribution varying from an altitude of 90 msl (Teesta Bazar) to 2487 msl (Senchal Lake) and its horizontal distribution from 27.0977° N, 88.8713° E (Bindu, Jaldhaka) to 27.1182° N, 88.1084° E (Rimbik, Darjeeling). In earlier study (Kumar et al., 2009), its presence was also noted at lower edge of Mahananda Wild Life Sanctuary. In all the surveyed areas, the population of this species has been found to be abundant. Thus, an immediate step has to be taken to control its invasive potentials. It has been invading to new places steadily and rampantly, thereby imposing a serious threat to the local flora as well.

Conclusion

Although, *Hypoestes phyllostachya* is an exotic element, it is believed to have escaped from some ornamental gardens and naturalized in some parts of India. By observing the rate of invasion of *Hypoestes phyllostachya* in Darjeeling and Kalimpong regions of Eastern Himalayas, its threat to the local flora is very clear. Moreover, the present study revealed that its vertical as well as horizontal distribution is quite extensive. Therefore, implementation of its control measure(s) is an immediate need of the hour.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 35-39

Research Article

Assessment of Growth Performance and Histochemical Localisation of Reactive Oxygen Species in Fenugreek under Exogenous Calcium Ion Priming

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Abstract

The objective of present study was to evaluate the response of the fenugreek seeds primed with various elicitors such as calcium chloride (CC) as an exogenous source of calcium ion; a calcium chelator: Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N, tetra acetic acid (EG); and Lanthanum chloride (LC): a calcium channel blocker, under salinity stress. Significant improvement in the growth parameters of fenugreek seedlings was observed comparing with control. The stress tolerance index (STI) and histochemical detection of reactive oxygen species were performed to evaluate the tolerance of the fenugreek against salinity stress. The results exhibited noteworthy inhibitory effect of salinity stress in control set which was significantly mitigated by the exogenous calcium ion application. Furthermore, under the influence of calcium ion antagonists, EG and LC the adverse effect of salinity was more prominent than control set. In conclusion present investigation revealed that exogenous calcium ion is an ideal elicitor for enhancing growth and development of the fenugreek with better salinity stress management.

Article info

Received **14 December 2022**
Revised **09 May 2023**
Accepted **21 May 2023**

Keywords: Calcium ion, Fenugreek, Salinity, Stress tolerance, Membrane integrity

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.006***

Introduction

The environmental factors are known to have significant impact on the morphological, biochemical attributes along with the growth and development of plants. When any of these factors exceed the tolerance level, a stress is imposed on the plant which influences its development and structural, physiological and biochemical processes (Imran et al., 2021). The increase in the salt content above optimum level, which creates salinity stress is considered one among these environmental factors which are responsible for threatening the crop productivity worldwide (Manivannan et al., 2007).

The deleterious effect of salinity which affects the normal growth and development of the plant is attributed to a reduced osmotic potential, specific ion toxicity and nutrient deficiency of the substratum (Nayem et al., 2020). The reduced osmotic potential affects water availability due to the prevention of water uptake by the plants, leading to a condition known as physiological drought (Kim et al., 2009). In addition, salinity is reported to result in the generation of reactive oxygen species which further leads to membrane disruption and metabolic toxicity in plant system (Mittler, 2002).

In plant system, calcium (Ca^{2+}) is considered as a key second messenger as well as signal transducer, which is involved in coupling a wide spectrum of extracellular stimuli to intracellular responses and play vital role in plant growth and development (Arshi et al., 2006). In the cited literature, Ca^{2+} is found to enhance tolerance against various environmental stresses, including salinity by mitigating oxidative stress and regulating membrane stabilization (Larkindale and Knight, 2002; Kader and Lindberg, 2010).

This study aimed to assess the potential calcium ion in improving growth performance and

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alleviating the detrimental effects of salt stress of fenugreek through priming technique.

Materials and method

Elicitation process and germination

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of 1mM calcium chloride (CC) as an exogenous source of calcium ion; a calcium chelator: 1mM of Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N, tetra acetic acid (EG); and 1mM of Lanthanum chloride (LC): a calcium channel blocker. For control set, seeds were primed with normal water and placed in a rotary shaker along with the treated seeds. After priming for 24h, the seeds were washed thrice with sterile water and kept in the seed germinator for germination. To provide saline conditions the NaCl at the level of (0dS m^{-1}, 4dS m^{-1} and 8dS m^{-1}) was applied to the seeds for 3days.

Measurement of growth parameters

The growth performance of the seedlings was assessed by calculating various morphological parameters. The average length of the roots and shoots of the seedlings of each experimental set up was recorded along with their fresh weight and dry weight.

Relative water content

The relative water content (RWC) of the seedlings was calculated using the following equation (Gonzalez and Gonzalez-Vilar, 2001):

RWC (%) = (Fresh weight - Dry weight) / (Turgid weight - Dry weight) x 100.

Stress tolerance index

The stress tolerance index (STI) of the seedlings was calculated using the following equation (Sen and Mandal, 2018):

STI = (DWs /DWc) × 100

where DWs: Dry weight of seedlings under stress and DWc: Dry weight of control seedlings

Histochemical detection

The histochemical detection of lipid peroxidation was performed according to Pompella et al. 24 (1987). Plasma membrane integrity of the seedlings was detected by the method suggested by Yamamoto et al. (2001). For detection of H_2O_2 localisation in the roots, the fenugreek seedlings were stained for about 40 to 45 min in potassium iodide/starch reagent (4% w/v starch and 0.1M potassium iodide solution) as reported by Olson and Varner, (1993). Superoxide localisation was detected by NBT method (Singh et al., 2009). Stained roots were photographed under a Nikon SLR camera (Model: D3200).

Statistical analysis

The statistical tools such as MS Excel 2007 (Microsoft, Redmond, WA, USA), DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy), Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) and Multivariate Statistical Package (MVSP 3.1) were used for statistical analysis of data.

Result and Discussion

Since germination being the initial stage of plant development defines the quality of yield and development of plant; therefore, the plants must be provided with best condition during initial stages for better germination. It has been well known that saline environment constrains the growth and development of plant by virtue of their adverse effect on the various physiological and biochemical processes, including osmolytes accumulation and metabolism along with the antioxidant enzyme system (Li et al., 2014). The basic criteria for a stress tolerant plant is said to be survived under stress are maintenance of biomass production, growth performance, elongation of root and accumulation of biochemical markers such as proline, soluble sugars, polyamines, amino acids and reduced level of lipid peroxidation (Juan et al., 2005).

As a result, it was observed that the growth performance of fenugreek seedlings was extensively affected by the saline condition as it was evident by the reduction growth parameters of the seedlings (Fig. 1). An important parameter, relative water content was calculated and it was observed to be 75.44% and 60.25% for unprimed seedlings, 81.88% and 75.24% for calcium chloride pre-treated, 68.02% and 58.02% for EGTA primed and 66.35% and 62.24% for lanthanum chloride at 4dS m^{-1} and 8dS m^{-1} salinity respectively (**Table 1**). The parameter such as relative water content has been considered as one of the vital factors for the

assessment of the extent of salinity induced effects and the degree of tolerance in plants towards stress environment. Our findings suggest the positive effect of exogenous calcium priming on the RWC of fenugreek seedlings under salinity stress was in agreement to previous studies on several plants (Khan et al., 2010). The seedlings subjected to calcium chloride priming exhibited minimal reduction in root elongation during saline stress, which is considered to be one of the major physiological parameter for salinity tolerance (Tari et al., 2015). Like calcium ion another signalling molecule, nitric oxide is also reported to mitigate salinity stress in plants (Ahmad et al., 2016). Furthermore, the stress tolerance index was significantly enhanced by the calcium chloride pre-treatment as compared to control, on the other hand the decline in tolerance index under the influence of calcium antagonist suggest potential role of calcium ion in providing tolerance to plant against salinity (**Table 1**).

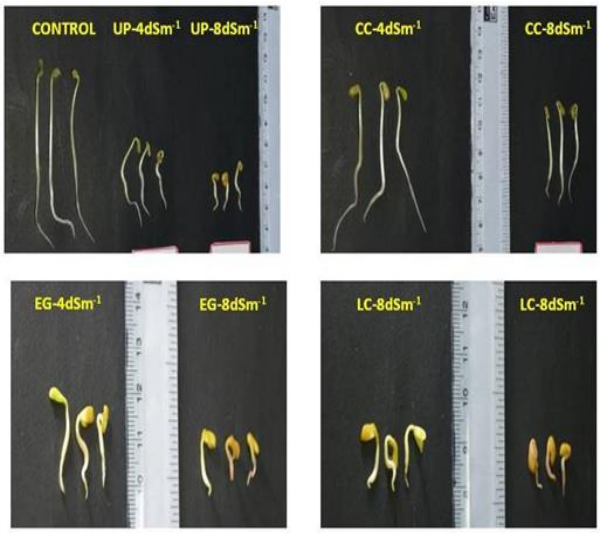

Fig.1 Fenugreek seedlings at various salinity level under influence of calcium elicitors

Our observation is in agreement with the reports of Joshi et al., (2013) which showed alleviation in salinity stress by calcium chloride priming in *Cucumis sativus*.

Table. 1 Effects of different treatments on the morphological attributes in fenugreek seedlings under saline conditions

Treatment	Salinity	Shoot length (cm)	Root length (cm)	Seedling length (cm)	RWC (%)	STI (%)
Control	4dS m^{-1}	2.03±0.05c	1.49±0.05b	3.52±0.17b	75.44±2.15b	81.84±2.02b
	8dS m^{-1}	1.67±0.09d	0.97±0.06d	2.64±0.19c	60.25±1.02d	61.32±1.05d
CC	4dS m^{-1}	2.42±0.11a	1.86±0.18a	4.28±0.13a	81.88±1.78a	88.13±1.12a
	8dS m^{-1}	2.16±0.09b	1.31±0.07c	3.47±0.15b	75.24±2.22b	78.25±1.02b
EG	4dS m^{-1}	1.55±0.08e	0.94±0.08d	2.49±0.16d	68.02±1.82c	65.25±1.26c
	8dS m^{-1}	1.08±0.04g	0.85±0.05e	1.93±0.11f	58.02±2.82d	51.25±1.14e
LC	4dS m^{-1}	1.33±0.11f	0.72±0.05f	2.05±0.11e	66.35±1.32c	62.42±1.24d
	8dS m^{-1}	0.95±0.06g	0.56±0.07g	1.51±0.05g	62.24±0.76d	54.26±1.76e

The major reactive forms of oxygen also termed as reactive oxygen species such as hydrogen peroxide, and superoxide radicals are known to be the molecules with high toxic potentials to plant tissues (Chen et al., 2022). The histochemical detection was performed for the study of specific localization of free radicals such as H_2O_2 and superoxide anion and their pattern of accumulation in the tissue. A major enhancement in the ROS generation both H_2O_2 (**Fig. 2A**) and superoxide radical (**Fig. 2B**) was observed, wherein the seedlings exposed to saline condition as well as those primed with the antagonists of calcium ion at considerably higher rate of accumulation was noted with respect to those primed with exogenous calcium.

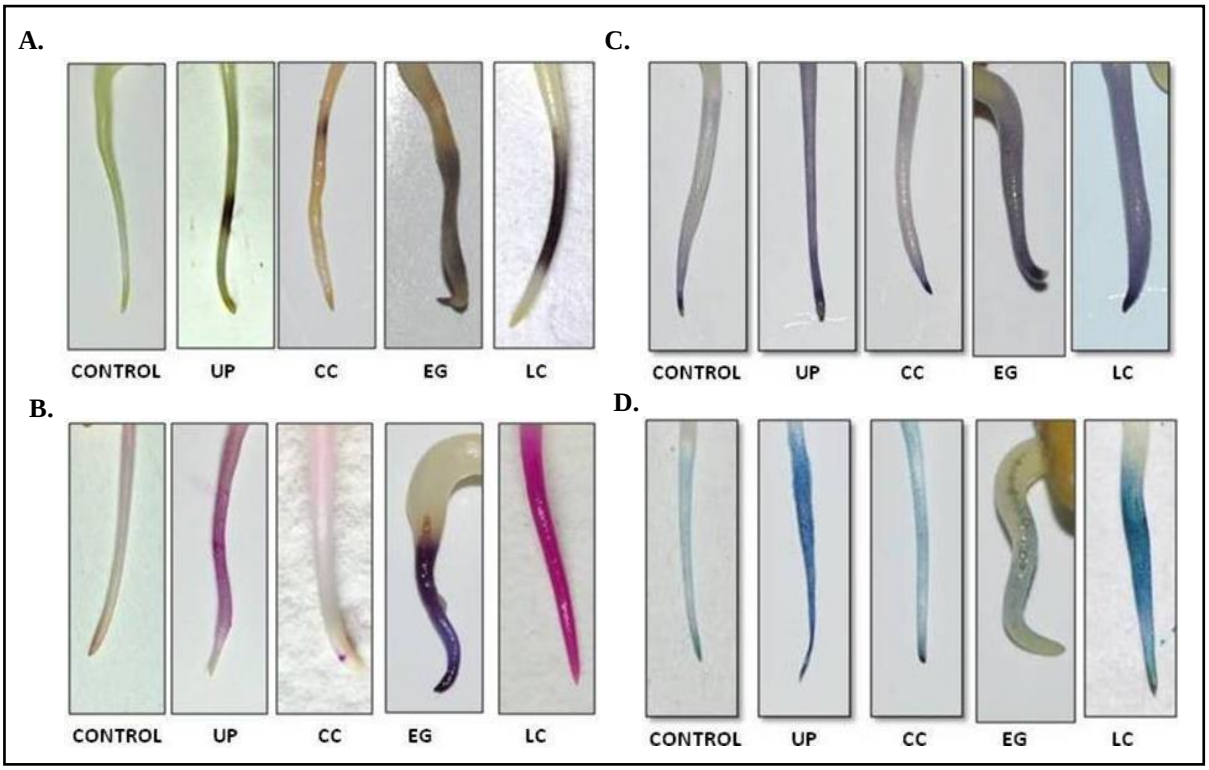

Fig. 2 Histochemical detection of ROS localisation in fenugreek seedlings under salinity stress. (A) Hydrogen peroxide, (B) Superoxide radical, (C) Lipid peroxidation and (D) Plasma Membrane Integrity.

The peroxidation of lipid bilayer of the cell membrane is one of the prominent adverse effects of salinity stress in biological system (Ahmad et al., 2014). Such damages and injuries of biological membranes due to lipid peroxidation are measured in terms of malondialdehyde content (Hogg and Kalyanaraman, 1999) which indicates the degree of stress experienced by the tissue. Consequently, the membrane fluidity is disrupted leading to ion leakage from the tissue. In agreement to aforesaid statement, it was observed that the degree of lipid peroxidation was found to increase considerably in response to salinity and further elevation was observed in the seedlings under the influence of antagonists as shown in Fig 2C. Similarly, the plasma membrane integrity was found to be affected by salinity and the antagonists (Fig. 2D). The seedlings pre-treated with calcium chloride were found to be resistant and exhibited high degree of membrane integrity in concomitant with low amount of ROS localization. Furthermore, in agreement with our result, the protective role of calcium ion is also found to be reported in other plant system under salinity stress condition (Bhattacharjee, 2009; Tian et al., 2015).

Conclusion

In conclusion, our results suggest that priming of fenugreek seeds with exogenous source of calcium enhanced the morphological and biochemical attributes under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seeds which were unprimed and also those primed with the antagonists of these signalling molecules.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 40-48

Research Article

Morphological and Anatomical Studies on Some Members of Araceae of North Bengal

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Abstract

The present study aimed to characterize eight edible members of the family Araceae on the basis of their morphological and anatomical characteristics. The petiole length, leaf size, and corm size varied widely across the studied members. Significant variations in the stomatal index, stomatal length, and width were observed, although all were of paracytic type with elliptical to circular shapes. Leaf epidermal cells were found to be polygonal or puzzle shaped. Mesophyll cells were non-distinguishable as palisade or spongy parenchyma. The root vascular bundles were found to be exarch, either arranged circularly or remaining scattered in the ground tissue. Pith was also observed, either large, centrally located, or scattered. Petiole anatomy showed scattered vascular bundles with collateral xylem and phloem in almost all the species, except Ol *Kochu*, Panchmukhi *Kochu*, and Ghot *Kochu*. Presence of tannins was observed in the petioles of all the species, whereas calcium oxalate crystals in the form of raphides were found to be present in some members. Water vessels, either large or small were found in the petioles of all the species. The number of parenchyma cells varied from 2 to 6 depending on the species. All the morphological and anatomical characteristics would help identify the eight members of Araceae and provide information for future studies with them.

Article info

Received **11 November 2022**
Revised **06 May 2023**
Accepted **25 May 2023**

Keywords: Araceae, Anatomy, Light microscopy, *Kochu*

DOI - ***https://doi.org/10.55734/nbujps.2022.v14i01.007***

Introduction

The family Araceae comprises many perennials, evergreen, herbaceous members with underground modified stems viz. rhizomes, tubers, and corms or climbing habits (Noltie, 1994). Most aroids usually prefer moist, well-drained, and nutrient-rich soil with frequent fertilization and warm temperature (nearly 16 ºC.). Most varieties are propagated through vegetative propagation, like leaf cuttings, stem cuttings, layering, or division. However, seed propagation can sometimes be capricious (Al-Eisawi, 1998; Feinbrun- Dothan, 1986). Aroids are rhizome-bearing perennial plants. Most of these plants have originated from the Mediterranean region but some are also found in Europe, Western to Central Asia, and Northern Africa. Corm size ranges from 8 inches to nearly 2 feet in height (20.5-60.5 cm). Leaves are alternate or apparently basal, usually petiolated with sheathing leaf bases. Various leave blade patterns like- linear, simple, pinnate, radiate, pedate with cordate to sagittate types are also observed. Inflorescences are of spadix type often subtended by the membranous cataphyllus spathe, containing bisexual or unisexual flowers, depending on species. The leafy spathe may be violet, white, yellow, or brown coloured and may even be sweet or sharply scented. Flowers are usually red to orange in colour. Bisexual flowers show tepals (0, 4, or 6), stamens (4 – 6), bilocular anthers, and 3 loculed ovary. In the case of unisexual flowers, male flowers

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are represented by single stamen of synandrous type (fused stamens, subsessile); female flowers consist of single ovaries (sometimes associated with a sterile staminode), commonly unilocular (sometimes with 3 or 4 locules), ovules 1-many per locule, positioned parietal, basal or apical. Spadix is sometimes sterile, merely a terminal appendage in nature (Noltie, 1994).

The Araceae family comprises 3500 species worldwide. In India, there are 25 genera and 187 taxa distributed mainly in North-Eastern India, Eastern Himalayas, and Western Ghats (Botanical Survey of India, 2019). Some of the commonly growing members of Araceae that have been consumed by the people of North Bengal include Colocasia sp., Alocasia sp., Xanthosoma sp., Ariopsis sp., Amorphophallus sp. Out of these species, some are cultivated and some are gathered from the wild. Colocasia sp. is an important crop growing in humid areas, and used as a vegetable (Al-Obeidi et al., 2022). It is considered an ancient crop based on geographical distribution and genetic origin (Matthews, 2014). They are perennial tropical plants with roots (height about 3 m), with green leaves having upwardly directed reticulate veins. Flowers are not distinctly visible due to their tiny size and are arranged compactly with a succulent spathe with a height of approximately 20 cm (al-Obeidi et al., 2022). Alocasia macrorrhizos also known as giant taro from the same family, is mainly used as an ornamental crop, but in a few countries (Western Samoa, Tonga, Wallis, etc.), it has been used as a food crop. Taro grows its corm above the ground up to 1 meter to 2 meters in length and 20 cm in diameter (Garcia et al., 2008). Amorphophallus is economically important as a good source of starch, and as traditional medicine and grows wild as well as in cultivated conditions in India, Sri Lanka, China, Japan, and Indonesia (Gholave et al. 2019). Xanthosoma is widely cultivated in Asia, Africa, and Oceania with about 124 species and valued for edible purpose. The plant is nutritionally rich with energy, protein, starch, macro- and micronutrients and vitamins (Boakye et al. 2018). However, the aroid members are very difficult to identify due to the great complexity in their morphological and anatomical characters.

In this context, our study aims to investigate and understand some Araceae members' morphological and anatomical variations found in the North Bengal region. Characterization of the Araceae members will help in the proper identification and documentation that will benefit future studies.

Materials and methods

Collection of plant samples

Local underutilized varieties of eight different aroid plant samples under study were collected freshly from three districts of North Bengal (Table 1). Ol, Mukhi, and Shola (black, white) Kochu were collected near Matigara, Darjeeling; Ghot Kochu was collected from Balurghat, Dakshin Dinajpur; Maan and Dudh Kochu were collected from Bhote patty, Jalpaiguri; and Panchmukhi Kochu collected from Dhupguri, Jalpaiguri region. All the collected varieties were submitted to the North Bengal University Herbarium (NBU) for proper identification. After this, all the samples were declared to be the family of Araceae.

Morphological parameters

All the samples were propagated in the experimental garden, and the morphological observations on their habit and corm were performed. The length of the petiole, corm size, and leaf size of the samples were measured. Five petioles of each sample were measured using a scale, and the average values were recorded.

Anatomical parameters

Leaf anatomy

Epidermal peels from the median portion of leaf lamina were obtained following the standard method (Metcalfe (1960); Arogundade and Adedeji (2016). The peels were stained with safranin, later mounted in dilute glycerine, and observed under microscope (MD-52A). The epidermal cell's shape, size, and number of stomata were measured. The stomatal index of the leaf surfaces was calculated using the formula:

$$\textbf{Stomatal Index} = \frac{S}{S+E} \textbf{ X 100}$$

Where S = Number of stomata and E = Number of ordinary epidermal cells plus the subsidiary cells in the same unit area.

Root anatomy

The transverse section of the roots was obtained with the help of a sharp blade and stained with safranin and cotton blue. The sections of roots were then passed through different concentrations of ethanol gradation (50, 60, 70, 80, 90, and 100%). The section was later mounted on 25% glycerine and observed

under light microscope (Leica DM LS2). Photomicrographs of the different root sections were taken under the 40X The Length and diameter of the sections were measured with the aid of an ocular and stage micrometre.

Table 1. List of the aroid species *(Kochu)* and their collection site

Local/common name	Scientific name	Collection area
Ol Kochu	*Amorphophallus paeoniifolius* (Dennst.) Nicolson.	Shivmandir, Matigara, Darjeeling
Ghot Kochu	*Xanthosoma* sp.	Balurghat, Dakshin Dinajpur
Mukhi Kochu	*Colocasia esculenta* (L.) Schott.	Shivmandir, Matigara, Darjeeling
Shola Kochu (White)	*Colocasia esculenta* (L.) Schott.	Matigara, Darjeeling
Shola Kochu (Black)	*Colocasia esculenta* (L.) Schott.	Matigara, Darjeeling
Panchmukhi Kochu	*Colocasia esculenta* (L.) Schott.	Dhupguri, Jalpaiguri
Maan Kochu	*Alocasia macrorrhizos* (L.) G. Don.	Bhote Patty, Maynaguri, Jalpaiguri
Dudh Kochu	*Alocasia* sp.	Bhote Patty, Maynaguri, Jalpaiguri

Petiole anatomy

The transverse section of the petioles was done by the same method as described in the above section.

Results and discussion

Morphological parameters

Length of the petiole

Petiole length of the varieties was found to be distantly different from each other (Figure 1). The length of the petiole ranged from 10 to 110 cm and the diameter of the petiole measured from 3 to 20 cm. The petiole size was found to be highest in Dudh Kochu (Length- 30 to 110 cm and diameter- 12 to 15 cm), whereas the smallest petiole was observed in Mukhi Kochu (Length- 10 to 30 cm, and diameter- 3 to 7cm). The petiole sizes indicated the growth pattern, corm size, and storage ability (calcium, raphides, tannins, acicular crystals), and meant for defense, and structural strength of the individual varieties (Saadi and Mondol, 2012).

Corm size

Corm size of all the varieties ranged from 7 to 100 cm in length and 5 to 8 cm in diameter (Figure 1). The highest corm size was observed in Maan Kochu (Length- 50 to 100cm and diameter- 15 to 20cm), and the smallest corm size was observed in Mukhi Kochu (Length- 7 to 15cm, and diameter- 5 to 8cm). Corm size basically depends on the soil nutrition as well as the sizes of the petiole, leaves, and age of the plants (Ravi et al., 2011).

	Plant habit	Corm	Corm size	Petiole size	Leaf size
Panchmukhi			L- 14 to 20 cm, B- 11 to 15 cm	L- 55 to 70 cm, D- 4 to 5 cm	L- 22 to 30 cm, B- 18-24 cm
Shola (White)			L- 50 to 60 cm, B- 9 to 13 cm	L- 60 to 80 cm, D- 7 to 10 cm	L- 25 to 30 cm, B- 15 to 22 cm
Shola (Black)			L- 20 to 30 cm, B- 6 to 10 cm	L- 30 to 40 cm, D- 6 to 8 cm	L- 15 to 20 cm, B- 10 to 15 cm
Ghot			L- 15 to 20 cm, B- 10 to 13 cm	L- 50 to 60 cm, D- 5 to 7 cm	L- 20 to 28 cm, B- 15 to 20 cm
Dudh			L- 12 to 70 cm, B- 10 to 15 cm	L- 30 to 110 cm, D- 12 to 15 cm	L- 15 to 35 cm, B- 7 to 25 cm
Maan			L- 50 to 100 cm, B- 15 to 20 cm	L- 40 to 90 cm, D- 15 to 20 cm	L- 30 to 60 cm, B- 25 to 50 cm
Mukhi			L- 7 to 15 cm, B- 5 to 8 cm	L- 10 to 30 cm, D- 3 to 7 cm	L- 5 to 18 cm, B- 5 to 12 cm
Ol			D- 7 to 20 cm	L- 30 to 70 cm, D- 10 to 15 cm	L-10 to 15 cm, B- 5 to 7 cm

Figure 1. Morphological observations on eight local varieties of *Kochu* collected and propagated in the experimental garden

Leaf size

The leaf size of all the varieties ranged from 5 to 60 cm in length and 5 to 7 cm in breadth (Figure 1). Out of eight varieties, the largest leaf size was observed in Maan Kochu (Length- 30 to 60cm and breadth- 25 to 50cm), and the smallest corm size was observed in Ol Kochu (Length- 10 to 15cm, and breadth- 5 to 7cm). Size of the leaves varied according to the age of the plants, size of the corms, and also in the nutritional condition of the soil (Ravi et al., 2011).

Anatomical parameters

Leaf anatomy

Both the upper and lower epidermis were found to be composed of parenchymatous cells arranged in a single row of uniseriate polygonal and puzzled cells (outer and inner tangential cells, stomatal cells, crystal cells) (al-Obeidi et al., 2022). The epidermal cells' outer and inner tangential walls were thick and straight. The stomatal complexes were found to be paracytic type, where the kidney-shaped guard cells were arranged parallelly with the stomatal aperture.

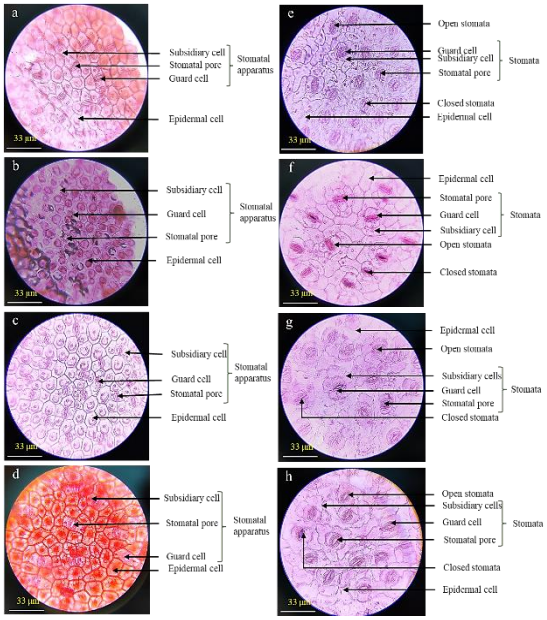

Figure 2. Leaf anatomy of eight varieties of Kochu under 40X – a) *Panchmukhi Kochu*, b) *Shola Kochu* (White), c) *Shola Kochu* (Black), d) *Ghot Kochu*, e) *Dudh Kochu*, f) *Maan Kochu*, g) *Mukhi Kochu*, h) *Ol Kochu*

Table 2. Quantitative attributes of the leaf anatomy of aroid varieties

Variety	Leaf anatomical parameters					
	Epidermal cell	*Stomatal shape*	*Stomata type*	*Stomatal Index*	*Stomatal Length*	*Stomatal Width*
Panchmukhi *Kochu*	Polygonal	Elliptical, Circular	Paracytic	27.8	13.55	8.32
Shola *Kochu* (white)	Polygonal	Elliptical, Circular	Paracytic	25.53	10.35	9.22
Shola *Kochu* (black)	Polygonal	Elliptical, Circular	Paracytic	20.65	14.65	7.26
Ghot *Kochu*	Polygonal	Elliptical, Circular	Paracytic	18.75	13.92	11.65
Dudh *Kochu*	Puzzle	Elliptical, Circular	Paracytic	28.94	18.63	14.12
Maan *Kochu*	Polygonal	Elliptical, Circular	Paracytic	17.14	17.32	11.83
Mukhi *Kochu*	Puzzle	Elliptical, Circular	Paracytic	26.38	18.85	15.25
Ol *Kochu*	Puzzle	Elliptical, Circular	Paracytic	31.03	20.65	14.21

The dimensions of the stomata also varied among the species as shown in Table 2. According to Suratman et al., (2016); and al-Obeidi et al., (2022), such variation of stomata depends on the surface area (both upper and lower) of the leaf. Raphides were found to be abundant, a characteristic feature of the family Araceae (Genua and Hillson, 1985). The mesophyll cells were not easily distinguishable as palisade and spongy parenchyma, as previously observed by Oluwabunmi et al. The xylem was composed of vessels arranged in rows of about 2-3 rows separated by parenchymatous cells.

Figure 2 and Table 2 showed that the stomatal index of Ol Kochu was the highest (31.03) and Maan Kochu's was the lowest (17.14) among all the studied varieties. However, the highest length of the stomata was found in Ol Kochu (20.65 µm) and lowest (10.35 µm) in Shola Kochu (white). On the contrary, Mukhi Kochu showed maximum stomatal width (15.25 µm), and the minimum stomatal width was observed in Shola Kochu (black).

Root anatomy

Transverse sections of the roots showed multilayered endodermis and pericycle composed of compactly arranged parenchymatous cells (Figure 3 and Table 3). The vascular bundle was found to be of exarch type, either arranged in a circular pattern (in Shola Kochu [white & black], Ghot Kochu, Dudh Kochu, Maan Kochu, and Mukhi Kochu) or scattered in the ground tissue (Panchmukhi and Ol Kochu). Pith was found to be conspicuous, made up of loosely arranged parenchymatous cells only in the).

varieties having vascular bundles arranged in a circular pattern. The cortex region consisted of many polygonal cell layers containing acicular calcium oxalate crystals (raphides). In some members like- Shola Kochu [white and black], chains of rectangular parenchymatous cells were found to be present that contain rosette crystals. Large air vacuoles were also found to be present in the cortical region of some members like- Shola Kochu (white and black

Table 3. Quantitative attributes of the root anatomy of aroid varieties

Variety	Root anatomical parameters					
	Vascular Bundle	*Pericycle layer*	*Parenchymatous cell*	*Aerenchymatos cells*	*Pith*	*Endodermis layer*
Panchmukhi *Kochu*	Exarch type, Scattered	2 to 3	Absent	Absent	Scattered	Multilayer
Shola *Kochu* (white)	Exarch type, Circular	2 to 4	Present	Present	Central, Small	4 to 6
Shola *Kochu* (black)	Exarch type, Circular	2 to 3	Present	Present	Central, Small	3 to 5
Ghot *Kochu*	Exarch type, Circular	2 to 3	Absent	Absent	Central, Large	Multilayer
Dudh *Kochu*	Exarch type, Circular	3 to 4	Absent	Absent	Central, Large	Multilayer
Maan *Kochu*	Exarch type, Circular	2 to 3	Absent	Absent	Central, Large	Multilayer
Mukhi *Kochu*	Exarch type, Circular	3 to 5	Absent	Absent	Central, Small	Multilayer
Ol *Kochu*	Exarch type, Scattered	3 to 4	Absent	Absent	Scattered	Multilayer

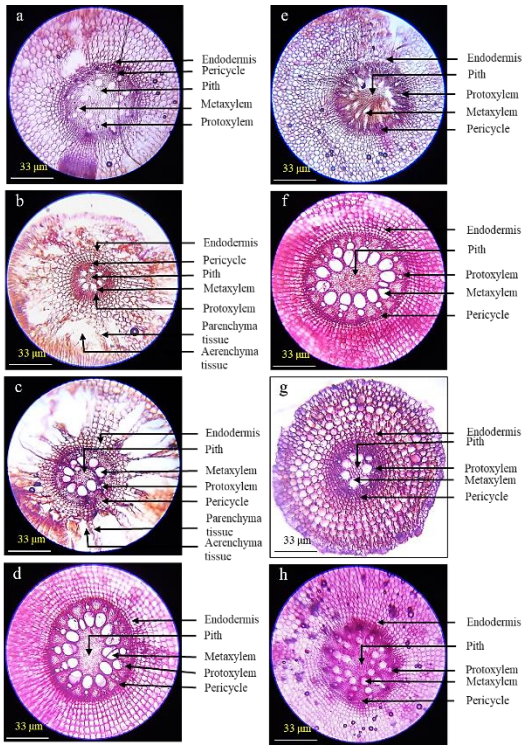

Fig. 3 Root anatomy of eight varieties of *Kochu* under 40X – a) Panchmukhi *Kochu*, b) Shola *Kochu* (White), c) Shola *Kochu* (Black), d) Ghot *Kochu*, e) Dudh *Kochu*, f) Maan *Kochu*, g) Mukhi *Kochu*, h) Ol *Kochu*

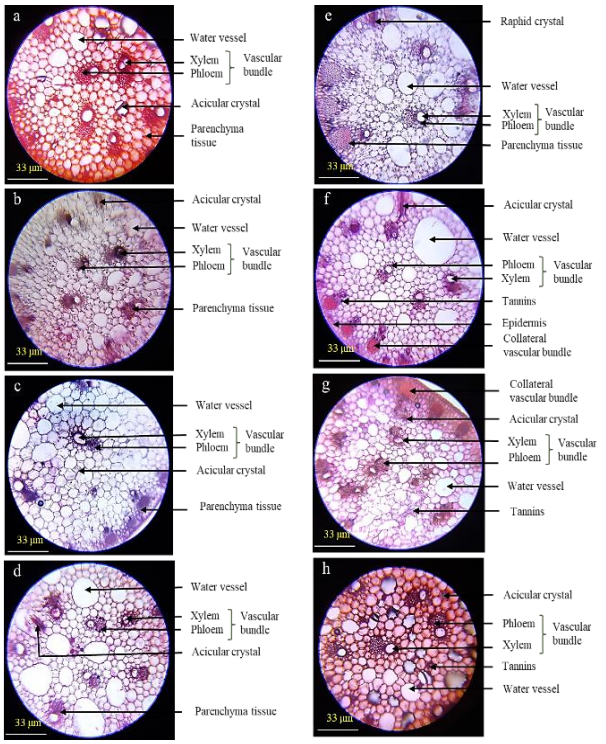

Fig. 4 Petiole anatomy of eight varieties of *Kochu* under 40X – a) Panchmukhi *Kochu*, b) Shola *Kochu* (White), c) Shola *Kochu* (Black), d) Ghot *Kochu*, e) Dudh *Kochu*, f) Maan *Kochu*, g) Mukhi *Kochu*, h) Ol *Kochu*

Petiole anatomy

All the anatomical data related to the petiole are included in Figure 4 and Table 4. The transverse section of the petiole is composed of layers from the outside to the inside of the epidermis of the layer of cells enveloping the petiole interspersed with parenchymatous cells, smooth layer of cuticle, scattered type of vascular bundle surrounded by a bundle sheath. Distinct protoxylem and metaxylem cavities were observed, which could be correlated with the previous observations by Suleiman (2003). Smaller water vessels were also found in Panchmukhi, Shola (black), and Mukhi Kochu varieties, whereas the larger vessels were observed in Shola (white), Ghot, Dudh, Maan, and Ol Kochu. Out of the eight varieties acicular crystals were absent in Ghot Kochu and raphides were absent in Shola Kochu (white), Mukhi Kochu, and Ol Kochu. Tannins were present in all the samples. Collateral vascular bundles with scattered distribution pattern were present in Shola Kochu [white & black], Dudh Kochu, Maan Kochu, and Mukhi Kochu), and Collateral vascular bundles with scattered distribution were absent in the varieties of Panchmukhi Kochu, Ghot Kochu, and Ol Kochu. Number of parenchymatous cells were observed to be in ascending order in the varieties of Mukhi and Dudh (3 – 6 cells), Maan (3 – 5 cells), Ol (3 – 4 cells), Panchmukhi and Shola Kochu [black] (2 – 4 cells), shola Kochu (1 - 4 cells), and Ghot Kochu (1 – 2 cells).

Conclusion

From the above results, it can be concluded that the eight members of Araceae vary significantly in terms of their morphological and anatomical characteristics. These data represent an essential basis for the differentiation of these species of the Araceae family. Further research is required to evaluate the physicochemical, biochemical, and other important properties that will help to characterize these locally available aroids for their nutritional benefits and commercial utilization.

Table 4. Quantitative attributes of the petiole anatomy of aroid varieties

Variety	Petiole anatomical parameters						
	Vascular Bundle	*Parenchymatous cells*	*Water Vessel*	*Acicular crystal*	*Raphides*	*Tannins*	*Collateral Vascular bundle*
Panchmukhi *Kochu*	Scattered type	2 to 4	Present, small	Present	Present	Present	Absent
Shola *Kochu* (white)	Scattered type	1 to 4	Present, Large	Present	Absent	Present	Present, Scattered
Shola *Kochu* (black)	Scattered type	2 to 4	Present, small	Present	Present	Present	Present, Scattered
Ghot *Kochu*	Scattered type	1 to 2	Present, Large	Absent	Present	Present	Absent
Dudh *Kochu*	Scattered type	3 to 6	Present, Large	Present	Present	Present	Present, Scattered
Maan *Kochu*	Scattered type	3 to 5	Present, Large	Present	Present	Present	Present, Scattered
Mukhi *Kochu*	Scattered type	3 to 6	Present, small	Present	Absent	Present	Present, Scattered
Ol *Kochu*	Scattered type	3 to 4	Present, Large	Present	Absent	Present	Absent

Acknowledgments

The first author acknowledges the fellowship received from the Govt. of West Bengal, India under the WBSVMCM V4.0 scheme (WBP211629121107). The authors also acknowledge the DST-FIST (2005-2009), Department of Botany, University of North Bengal for light microscopy.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 49-60

Research Article

Concept of The Genus *Acampe* (Lindl.) in India

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Abstract

The genus *Acampe* Lindl. (Orchidaceae) has been taxonomically studied in Indian context. Out of 10 species of *Acampe* found globally, four species and one variety were recognized in India. Detailed taxonomic account including description, illustrative photo-plate along with information on phenology, habitat and distribution have been provided. The threat status of each taxon has been assessed in Indian perspective by following the IUCN guidelines.

Keywords: *Acampe*, Conservation, Distribution, IUCN, Orchidaceae, Taxonomy

DOI: *https://doi.org/10.55734/nbujps.2022.v14i01.008*

Article info

Received **17 December 2022**
Revised **17 May 2023**
Accepted **25 May 2023**

Introduction

The genus *Acampe* [Family: Orchidaceae; Sub-family: Epidendroideae; Tribe: Vandeae; Sub-tribe: Aeridinae (Chase et al. 2015)] was established in 1853 by John Lindley in Folia Orchidacea. But Garay (1972) pointed out Lindley's earlier name *Sarcanthus* (typified by *Epidendrum praemorsum* Roxb.) had priority. Subsequently, there has been considerable difference in opinion regarding the application of names (*Sarcanthus*, *Saccolabium*, *Gastrochilus* etc.) for species of Acampe. Presently, the name *Acampe* has been conserved.

The generic name derived from the Greek word *akampes* in reference to the rigid, brittle flowers. The genus represents epiphytic habit with monopodial growth; leaves distichous, coriaceous with bilobed apex; inflorescence axillary, racemose, corymbose or paniculate; flowers non-resupinate, fleshy; labellum attached with the base of the column, rigid, papillose or sometimes warty (Pearce & Cribb, 2002; Pridgeon et al., 2014).

The genus comprises 10 taxa globally and is distributed in tropical Himalayan region to China and Indochina, Southeast Asia, tropical and subtropical Africa, Madagascar and the island of the western Indian Ocean (Pridgeon et al., 2014). In India, it is represented by four species and one variety [viz. *Acampe ochracea* (Lindl.) Hochr; *Acampe papillosa* (Lindl.) Lindl.; *Acampe papillosa* var. flava A.P. Das et al.; *Acampe praemorsa* (Roxb.) Blatt. & McCann; *Acampe rigida* (Buch. -Ham. ex Sm.) P.F. Hunt]. Singh et al. (2019) included *Acampe congesta* Lindl. (Lindl.) also, but this has been reduced under the synonymy of *Acampe praemorsa* during the present study. The genus is predominantly distributed almost throughout India in the tropical and coastal region.

As a part of the revisionary studies of monopodial orchids under the Himalayan Research Fellowship scheme of NMHS, the genus *Acampe* was studied in detail. The taxonomic study was based on the thorough morphological characterization of each taxon through study of fresh and herbarium specimens coupled with study of relevant literature. Each taxon has been provided with detailed description, photographic illustrations, information on phenology, habitat and distribution. An artificial key for identification of all the species of *Acampe* present in India has been provided. Threat status for each taxon has been assessed in accordance with IUCN guidelines (IUCN, 2012a; 2012b; 2019) in order to facilitate conservation actions.

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Taxonomic Treatment

Acampe (Lindl.) Lindl., Fol. Orchid. Acampe: 2. 1853; Benth. & Hook.f., Gen. Pl. 3: 579. 1883; Bose & Bhattacharjee, Orchids India: 49. 1980; N.P. Balakr., Fl. Jowai: 473. 1983; Deb, Fl. Tripura 2: 448. 1983; Kataki, Orch. Meghalaya: 181. 1986; Karthik. & al., Fl. Ind. Enum. Monocot.: 106. 1989; H.J. Chowdhery, Orch. Fl. Arunachal Pradesh: 39. 1998; Sushil K. Singh & al., Orch. India: 35. 2019. **Type:** *Acampe multiflora* (Lindl.) Lindl. [**=*Acampe rigida*** (Buch. -Ham. ex J.E. Sm.) P.F. Hunt]. *Saccolabium* Bl. sect. (Acampe) Hook.f., Fl. Brit. India 6: 32.1890; King & Pantl., Ann. Roy. Bot. Gard. Calcutta 8:217. 1898. *Sarcanthus* Lindl., Bot. Reg., 10, t. 817 (1824), non. Lindl. (1826).

Epiphytic monopodial herb; roots thickly velamenous; stem woody, simple or branched. Leaves distichous, coriaceous, dorsiventrally flattened, fleshy, apex bilobed. Inflorescence axillary, simple or branched, racemose, corymbose or paniculate, much shorter or sometimes longer than leaves. Floral bracts membranous, brownish. Flowers small to medium sized, non-resupinate, rigid and fleshy, yellowish with brown transverse bands. Sepals and petals sub-similar, labellum saccate or spurred at base; immobile, rigid, lobed, fleshy, papillose or sometimes warty; spur non septate, lacking back wall callus but hairy inside. Column erect, short, stout, fleshy, glabrous or papillose, foot absent; anther cap ovate; pollinia 4 in unequal pairs, globose waxy; stipe strap shaped, longer than wide; viscidium small, ovoid; rostellum emarginate. Capsules erect, fusiform-clavate, ridged, stalked.

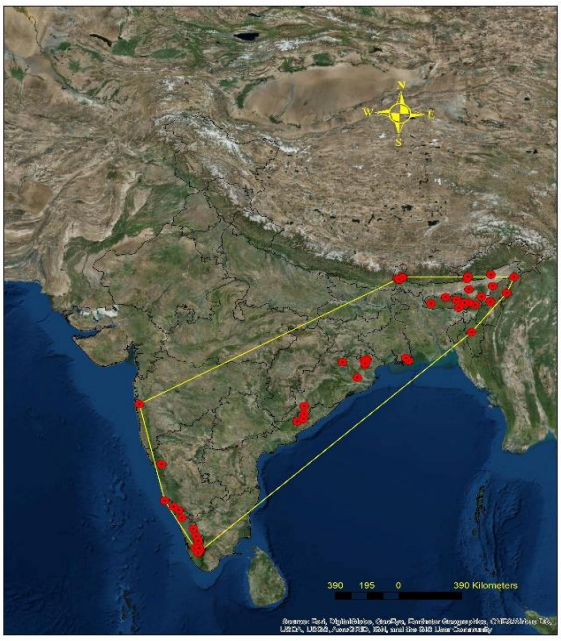
Notes: Some species are often cultivated as ornamentals. There are reports of few species being used in traditional medicine.

Key to the species

1a. Mid-lobe of the labellum warty-papillose, spur 3 – 4 mm long, cylindrical **2**
1b. Mid-lobe of the labellum finely pubescent, spur sac like, 1 – 2 mm long, broadly conical **3**

2a. Inflorescence paniculate or simple raceme, equal to or longer than leaves; side-lobes of labellum small, protruding at base, column with two distinct horns at apex 1. ***A. ochracea***
2b. Inflorescence umbellate to capitate, not branched, shorter than leaves, side lobes of labellum not protruding, column without prominent horns at apex 2. ***A. papillosa***

3a. Plant up to 70 cm long; leaves 2 – 3 cm wide; side-lobes of labellum reduced, truncate; disc without central pubescent ridge 3. ***A. praemorsa***
3b. Plant up to 150 cm long; leaves 2.5 – 5.5 cm wide; side-lobes of labellum well developed, broadly conical; disc with a central pubescent ridge 4. ***A. rigida***



Map 1 Distribution map of *Acampe ochracea* (Lindl.) Hochr.

1. *Acampe ochracea* (Lindl.) Hochr. in Bull. New York Bot. Gard. 6: 270. 1910; Pradhan, Indian Orchids 2: 525. 1979; A. Abraham and Vatsala, Intr. Orchids: 452, f. 136. 1981; B.D. Sharma & al., Fl. Karnataka: 264. 1984; Kataki, Orch. Meghalaya:182, Pl.67 (1a-c). 1986; Seidenf. in Opera Bot. 95: 48, f.25. 1988; Karthik. & al. in Fl. Ind. Enum. Monocot.: 106. 1989; Lakshmin. in B.D. Sharma & al. (ed.). Fl. Maharashtra: 10. 1996; K.N. Ramakrishna in Pull. (ed.), Fl. Andhra Pradesh 3: 931. 1997; H.J. Chowdhery, Orch. Fl. Arunachal Pradesh: 39, f.11. 1998; Hynn. & al. in Hajra & U. Chatterjee (eds.), Orch. Nagaland: 27. 2000; N. Pearce & P.J. Cribb, Fl. Bhutan 3(3): 489. 2002; S. Misra, Orch. Orissa: 577. 2004; C.S. Kumar & Manilal in Manilal & C.S. Kumar (eds.), Orchid memories: 163. 2004; Lucksom, Orch. Sikkim: 807. f.487. 2007; K. Gogoi, Wild Orch. Assam: 27. 2017; P.G. Diwakar in Lakshmin. & al. (eds.), Fl.

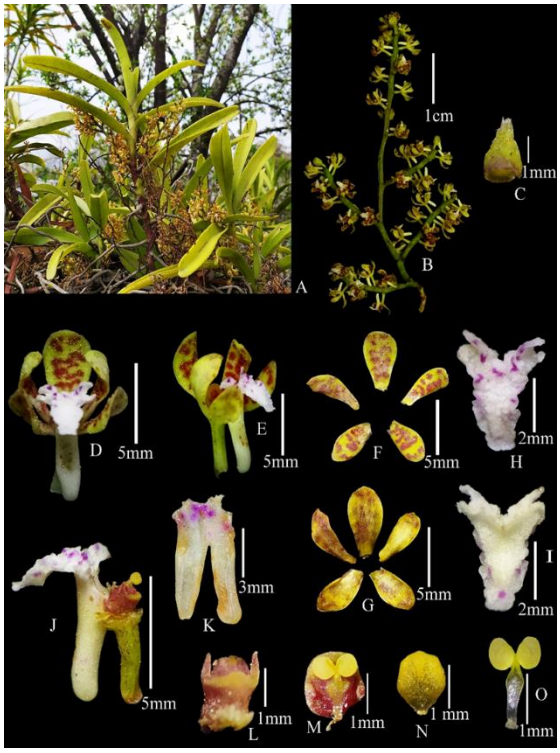


Fig. 1 *Acampe ochracea* (Lindl.) Hochr.: A. Habit; B. Inflorescence; C. Bract; D. Flower (front view); E. Flower (side view); F. Sepals & petals (ventral view); G. Sepals and petals (dorsal view); H. Labellum (ventral view); I. Labellum (dorsal view); J. Labellum with column, pedicel and ovary; K. L.S. of spur; L. Column; M. Column with pollinia; N. Anther cap; o. Pollinia.

Karnataka 3: 12. 2019; Sushil K. Singh & al., Orch. India: 35. 2019. **Type:** Sri Lanka (Ceylon), Horton, cult. *Loddiges* s.n. (holo. K- LINDL, photo!). *Saccolabium ochraceum* Lindl., Bot. Reg. 28: misc.2, no.4. 1842; Hook. f. Fl. Brit. India 6: 62. 1890p.p.; King & Pantl., Ann. Roy. Bot. Gard. Calcutta 8: 219, t. 291. 1898; Prain, Bengal Plants: 768. 1903. *Acampe dentata* Lindl., Fol. Orchid. 4 (Acampe): 3. 1853. *Saccolabium lineolatum* Thwaites, Enum. Pl. Zeyl.: 304. 1864. *Acampe griffithii* Rchb.f. in Flora 55: 277. 1872. *Gastrochilus ochraceus* (Lindl.) Kuntze., Revis. Gen. Pl. 2: 661. 1891.

Epiphytic monopodial herbs, up to 1 m tall; stem 0.9–1.0 cm thick; erect to ascending, enclosed in persistent, tubular, imbricate, finely striated leaf sheaths. Leaves alternate, distichous, coriaceous, 10–25 × 1.0–2.5 cm, linear-oblong, entire, apex unequally bilobed, mid-vein prominent, sheathed at base. Inflorescence paniculate or simple raceme, 7–26 cm long, lateral, extra axillary, equal to or longer than leaves, rachis laxly 8–20 flowered. Floral bracts 1.1–1.4 × 0.9–1.0 mm, minute, ovate-triangular, fleshy. Flowers widely opening, 6–7 mm across, yellow with irregular brown markings at sepals and petals, labellum white with creamy-yellow spur. Pedicel and ovary 4 mm long, cylindrical, sparsely puberulous. dorsal sepal 5.5–6 × 1.5–2.5 mm, oblanceolate, obtuse; lateral sepals 5.0–5.6 × 1.3–2.5 mm, oblong, sub-falcate, obtuse; petals c. 5 × 2 mm, oblanceolate-spathulate, obtuse. Labellum 3.5–4.5 × 2–2.5 mm, 3-lobed, fleshy, distinctly spurred; side lobes small, erect, toothed, protruding at base; mid-lobe broadly oblong-obtuse, warty adaxially, conical projection at the abaxial side, margins undulate; spur cylindrical, 3–4 mm, hairy within. Column 1.5 mm long, with two distinct horns at apex, finely puberulous; anther cap sub-orbicular, convex with recurved beak; pollinia 4, sub-globose, cleft, stipe 1 mm, sub-clavate; viscidium small, glandular. Capsules 3.0–5 × 0.4–0.6 cm, fusiform, ridged, stalk small.

Phenology: September – March.

Habitat: Epiphytic herbs; grow on moss covered branches and rock boulders in valleys and tropical-subtropical forest at elevation up to 1500 m.

Distribution: India (Andhra Pradesh, Arunachal Pradesh, Assam, Karnataka, Kerala, Maharashtra, Manipur, Meghalaya, Mizoram, Nagaland, Odisha, Sikkim, West Bengal); Bangladesh; Bhutan; China; Indo-China; Myanmar; Nepal; Sri Lanka; Thailand.

Exsicata: Andhra Pradesh: Ebul R. F., North of Gulium, 23.02.47, *V. Narayanaswami & Party* 570 (CAL). **Arunachal Pradesh:** West Kameng, Tipi Orchid Centre, 29.11.2000, *A.N. Rao* 30616 (OHT). **Assam:** Nowgong, Khulahat Forest, 24.11.1952, *G.K Deka* s.n. (ASSAM); Pava Sanctuary, Rajabari, 03.04.1976, *Hajra* 52585 (ASSAM). **Kerala:** Pathanamthitta, Angamoozhy, 28.02.1988, *Anil Kumar. N.* 528 (CAL); Ganapathy Temple area, Pamba Sabarimala R.F, 25.04.1984, *E. Vajravelu* 80584 (CAL); Idukki, Adimala, 20.03.1991, *A. Gangaprasad* 12221 (TBGT). **Manipur:** Willong, 02.03.2011, *Nanda* 00174 (COGCEHR). **Meghalaya:** Jaintia Hills, Jowai, Raliang, 24.01.1957, *G.K Deka* 5130 (ASSAM, CAL); Garo Hills, 10 mile down to Dalu Road, 19.12.1950, *M.L. Saikia* 36177 (ASSAM). **Mizoram**: Aijawl to Sibutalung road, 19.02.1953, *G.K. Deka* s.n. (ASSAM). **Nagaland**: Tisu, *Hynniewta* 80848; Tokye, *Hynniewta* 80915 (ASSAM). Naga hills, *F. Kingdon Ward* 11208 (CAL). **Odisha**: Mayurbhanj, Simlipal, Barigam-Kabataghai, 05.01.1987, *S. Misra* SM1042 (CAL). **Sikkim:** Tropical Valley, November 1895, *Pantling* 133 (CAL). **Tripura:** *s. loc*., *D.B. Deb s.n.* (CAL). **West Bengal**: Jeodhara, 01.08.1900, *Prain* s.n. (CAL).

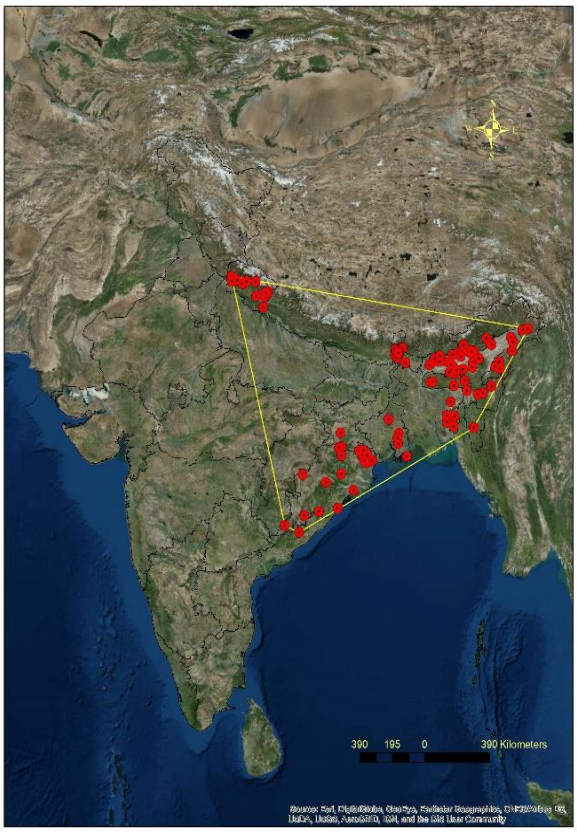
Extent of occurrence (EOO): 1889346 km^2

Area of Occupancy (AOO): 228 km^2

Red list assessment: Endangered [EN B2ab(iii)+C2a(i)].

Final assessment in Indian perspective: Vulnerable° [VU°].

Uses: This species has been cultivated at some commercial Orchid nurseries and got good potential for a potted ornamental for foliage and can be hanged in lobbies and verandas.



Map 2 Distribution map of *Acampe papillosa* (Lindl.) Lindl.

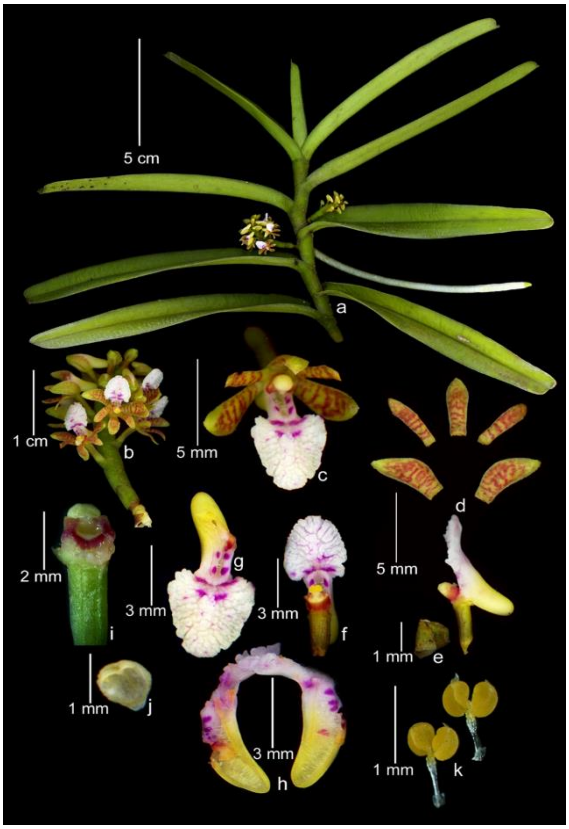


Fig. 2 *Acampe papillosa* (Lindl.) Lindl.: a. Habit; b. Inflorescence; c. Flower; d. Floral parts showing lateral and dorsal sepals, petals, spurred labellum with column; e. Bract; f & g. Labellum; h. Section of spur; i. Column; j. anther cap; k. Pollinia.

2. *Acampe papillosa* (Lindl.) Lindl., Fol. Orch. (Acampe): 2. 1853; Pradhan, Indian Orchids 2: 525. 1979; Deb, Fl. Tripura 2: 448. 1983; Kataki, Orch. Meghalaya: 183, pl.67 (2a-b). 1986; Seidenf., Opera Bot. 95: 49, f.26. 1988; Karthik. & al., Fl. Ind. Enum. Monocot.: 106. 1989; H.J. Chowdhery, Orch. Fl. Arunachal Pradesh: 41, f.12. 1998; Hynn. & al. in Hajra & U. Chatterjee (eds.), Orch. Nagaland: 28. 2000; N. Pearce & P.J. Cribb, Fl. Bhutan 3(3): 491, f.106. 2002; Lucksom, Orch. Sikkim: 807, f.482, pl.35. 2007; H.J. Chowdhery & Agrawala, Cent. West Himalayan Orch.: 82, pl.1. 2013; K. Gogoi, Wild Orch. Assam: 138. 2017; Sushil K. Singh & al., Orch. India: 35. 2019. **Type:** Myanmar (Burma), Prome, coll. *Wallich* Wall. Cat. 7305 (holo. K-Lindl., photo!). *Saccolabium papillosum* Lindl., Bot. Reg. 18: t.1552. 1833 (excl. syn.); Hook.f. Fl. Brit.India 6: 63. 1890 (excl. syn. *Sarcochilus praemorsus* Spreng.); King & Pantl., Ann. Roy. Bot. Gard. Calcutta 8: 219, t. 290. 1898; Prain, Bengal Plants: 768. 1903; Duthie, Ann. Roy. Bot. Gard. Calcutta 9: 147. 1906; Haines, Bot. Bihar Orissa 6: 1180. 1924; Panigrahi & J. Joseph in Bull. Bot. Surv. India 8(2): 157. 1966. *Saccolabium carinatum* Griff., Not. Pl. Asiat. 3: 354. 1851. *Gastrochilus papillosum* (Lindl.) Kuntze, Rev. Gen. Pl. 2: 661. 1891; A.S. Rao & N.P. Balakr., Rec. Bot. Surv. India 20(2): 85. 1973. *Sarcanthus papillosus* (Lindl.) Tixier, Bull. Soc. Roy. Sci. Nat. Laos 9: 30. 1963. *Acampe carinata* (Griff.) Panigrahi, Taxon 34(4): 688. 1985; Deva & H.B. Naithani, Orch. Fl. NW Himalaya: 355, f. 203. 1986; S. Misra, Orch. Orissa: 580. 2004; H.A. Barbhuiya & al., Phytotaxa 303(3): 272, f.1. 2017 (excl. syn. *Acampe papillosa* var. *flava*). *Acampe praemorsa* auct. non (Roxb.) Blatt. & McCann: Guha Bakshi, Fl. Murshidabad district,

Key to the varieties

1. Flowers large, floral parts less fleshy, sepals pale green and marked with brown streaks on the lower side and brown blotches at the apical end; petals pale green with horizontal brown steaks and blotches at the tip; labellum white with dark spots at the base, ovate-cordate, strongly papillate throughout **2.1. *A. papillosa* var. *papillosa***

1a. Flowers small, floral parts much fleshy & shinning, flowers evenly yellow throughout markings or blotches obscure; labellum pale white, **2.2. *A. papillosa* var. *flava***

West Bengal: 310. 1984; D.K. Ghosh & J.K. Mallick, Fl. Darjeeling Himalayas: 653. 2014; K. Gogoi, Wild Orch. Assam: 28. 2017.

Epiphytic, monopodial herbs up to 45 cm; stem erect to ascending; internodes 1–3 cm long, enclosed in persistent, tubular, imbricate sheaths. Leaves distichous, coriaceous, 13–16 × 1.0–2 cm, linear-oblong, apex obliquely notched, midvein prominent. Inflorescence lateral, extra-axillary, sub-umbellate to capitate, much shorter than leaves, densely many flowered; peduncle with 1-2 annular sheaths. Floral bracts 1–1.5 mm, ovate-triangular, acute. Flowers 10–12 mm across, yellowish-green with dark purple bands and spots on sepals and petals, labellum white with dark purple spots towards the base. Pedicel plus ovary 4–5 mm long, glabrous, ridged. Dorsal sepal 5–7.5 × 1.5–2 mm, oblong-oblanceolate, entire, obtuse; lateral sepals 5–8 × 2–2.5 mm, oblong-elliptic, sub-acute, falcate; petals 5.0–7.5 × 1–1.3 mm, oblong-elliptic, sub-acute, falcate. Labellum 6 × 4.1 mm, 3-lobed, base concave; side-lobes much reduced, not protruding out; mid-lobe 4–6 × 2.5–3 mm, large, ovate-cordate, strongly papillate throughout, obtuse, undulate, decurved; spur 3 mm long, cylindrical, pubescent at the opening and inner margin, parallel to the pedicel and ovary. Column c.1.5 mm long, short, stout, sparsely puberulous, stelidia obscure; anther cap ovate; pollinia 4, globose, in two unequal pairs; stipe 0.5 mm, linear, viscidium small, glandular. Capsules fusiform, 1.8–4 × 0.4–0.5 cm, fusiform, ridged, stalk small.

Phenology: October – June.

Habitat: Epiphytic herbs; on rough surfaced trees in moist deciduous and semi evergreen forests, often on roadside trees found at elevation up to 1500 m.

Distribution: India (Andhra Pradesh, Arunachal Pradesh, Assam, Bihar, Chhattisgarh, Jharkhand, Madhya Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Odisha, Sikkim, Tripura, Uttarakhand, West Bengal); Bangladesh; Bhutan; China; Indo-China; Myanmar; Nepal; Thailand.

Exsicata: Arunachal Pradesh: Lohit, near Chowkham, 24.11.1969, *J. Joseph* 48317 (ASSAM); Parshuram Kund, 01.01.1970, *J. Joseph* 48857 (ASSAM); West Kameng, Tipi, 12.11.1979, *S.N. Hegde* 527 (OHT); Tipi, 2.10.1993, *A.N. Rao* 21841 (OHT). **Assam:** Bornadi WLS, Udalguri & Baksa, Bogamati, 20.09.2010, *C. Deori & D.K. Roy* 19070 (ASSAM); Sonitpur, Sonai Rupai WLS, 11.04.1976, *Hajra* 65459 (ASSAM). **Chhattisgarh:** Surguja, 30.08.1901, *Rev. L. Candom* s.n. (CAL); 23.07.1902, *Rev L. Candom* s.n. (CAL). **Jharkhand:** Chotanagpur, 13.09.1896, *Prain* s.n. (CAL). Jaintia Hills, Raliang-Jowai, 24.01.1957, *G.K. Deka* 5130 (ASSAM). **Manipur:** Imphal East District, Jiri, 08.11.12, 13.10.11, *Nanda et al* 00294, 00482 (COGCEHR). **Meghalaya:** Khasi & Jaintia Hills, Bornihat, 26.03.1950, *G.K. Deka* (ASSAM); Ri-Bhoi, Nongpoh, 27.10.1935, *G.K. Deka 20269* (ASSAM); Garo Hills, *Parry* 911, 1013 (K). **Nagaland:** Mokokchung, *Hynniewta* 80399, Wokha, *Hynniewta* 80644 (ASSAM). **Odisha:** Mayurbhanj, Similipal, Bhanjbasa, 13.02.1958, *G. Panigrahi* 12342 (ASSAM); Raipani (Kaptipada-Savat), 18.09.1990, *S. Misra* 1680 (CAL). **Sikkim:** Tropical Valleys, Sep 1892, *Pantling* 243 (CAL, BM, K, W). **Tripura:** Sonamura, 15.02.57, *D.F.O. Tripura* 584 (CAL); Teliamura, 23.04.65, *D.B. Deb* 2887 (CAL); Kalasi, 29.12.59, *D.B. Deb* 2158 (CAL); Rangamura, 08.12.14, *P.M. Deb barman* 600 (CAL); Pratapgarh, South of Agartala, 29.12.14, *P.M. Deb barman* 449 (CAL). **Uttarakhand:** Dehradun, Mussoorie, 08.12.1903, *Mackinnon* (CAL); *Anonym*. 23292 (CAL, K); Lachiwala *Deva* 8835, 30.01.1962, *Malhotra* 19753 (BSD); 25.06.1966, *Arora* 36458 (BSD); Kumaon, *Inayat* 24126 (K); Pithoragarh, Tuli-Baram, G.G. Valley, 21.06.1982, *Malhotra* 51521 (BSD). **West Bengal:** Darjeeling, Mahananda WLS, Sukna, 23.11.2006, *T.K. Paul & Anant Kumar* 41295 (CAL); West Bengal, Kalimpong, Holumba Nursery, 16.12.2003, *G. M. Chhetri* 19814 (BSHC).

Extent of occurrence (EOO): 1444363 km^2

Area of Occupancy (AOO): 524 km

Red list assessment: Vulnerable [VU C2a(i)].

Final assessment in Indian perspective: Near Threatened° [NT°].

Notes: Panigrahi (1985) noted the name *A. papillosa* was illegitimate and proposed the new name *A. carinata* (Griff.) Panigrahi. Seidenfaden (1988) discussed this change and proposed an alternative solution which preserved the older, more well established, name and that has been followed here.

2.2. *Acampe papillosa* (Lindl.) Lindl. **var**. ***flava*** Das, Katham & Nirola, Pleione 4(1): 155, f.1. 2010; Sushil K. Singh & al., Orch. India: 35. 2019. **Type:** Nagrakata, Duars, Jalpaiguri, 17.11.2009; *AP. Das &T K. Katham* 4193 (NBU, not seen).

Map 3 Distribution map of *Acampe papillosa* var *flava* A.P. Das et al

Monopodial epiphytic herbs; stem erect to ascending; internodes 1–3 cm long, enclosed in persistent, tubular, imbricate sheaths. Leaves distichous, coriaceous, 8–15 × 1.0–2 cm, linear-oblong, apex obliquely notched, midvein prominent. Inflorescence extra-axillary, sub-umbellate to capitate, much shorter than leaves; peduncle 1–2 cm, with purplish-brown annular sheaths; rachis densely many flowered. Floral bracts 1–1.5 mm, ovate-triangular. Flowers 10–13 mm across, faintly fragrant, fleshy and shining, uniformly yellow throughout without any band or blotch; labellum creamy white with yellowish flush on disc and mid-lobe; spur pale greenish-yellow. Pedicel and ovary 4–5 mm long. Dorsal sepals 5–6.5 × 1.5–2, oblong-oblanceolate, obtuse; lateral sepals 5–6.5 × 2–2.5 mm, oblong-elliptic, sub-acute, weakly falcate. Petals 5–6.3 × 1–1.5 mm, linear-elliptic, sub-acute. Labellum 4.5–6 × 3–4 mm, obscurely 3-lobed; side-lobes erect, not protruding out; mid-lobe ovate, deflexed, obtuse, erose-undulate, surface densely papillose; spur 2.5 mm long, cylindrical-conical, white-pubescent inside.

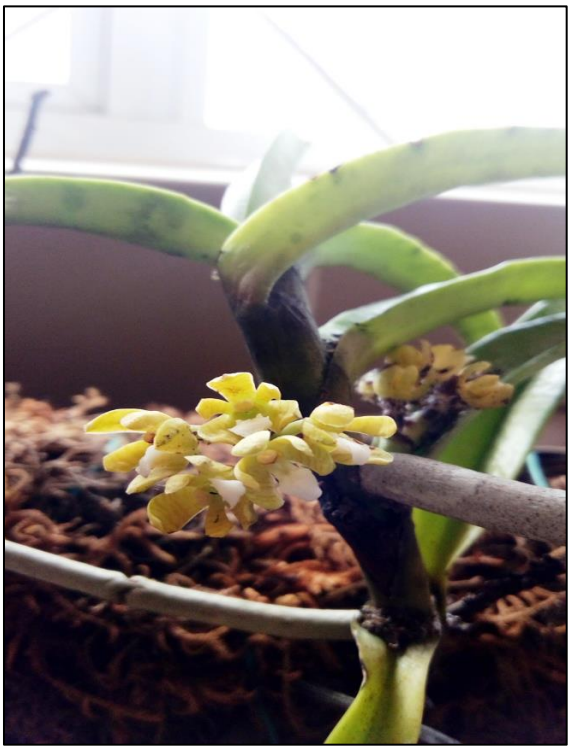


Fig. 3. Inflorescence of *Acampe papillosa* var *flava*. A.P. Das et al.

Phenology: Flowering: August – November.

Habitat: Epiphytic in moist evergreen forest up to 400 m.

Distribution: India (West Bengal), ENDEMIC.

Exsicata: India: West Bengal: Jalpaiguri, Nagarkata, 17.11.2009, *A.P. Das & T.K. Katham* 4193 (NBU, could not be located).

Area of Occupancy (AOO): 08 km^2.

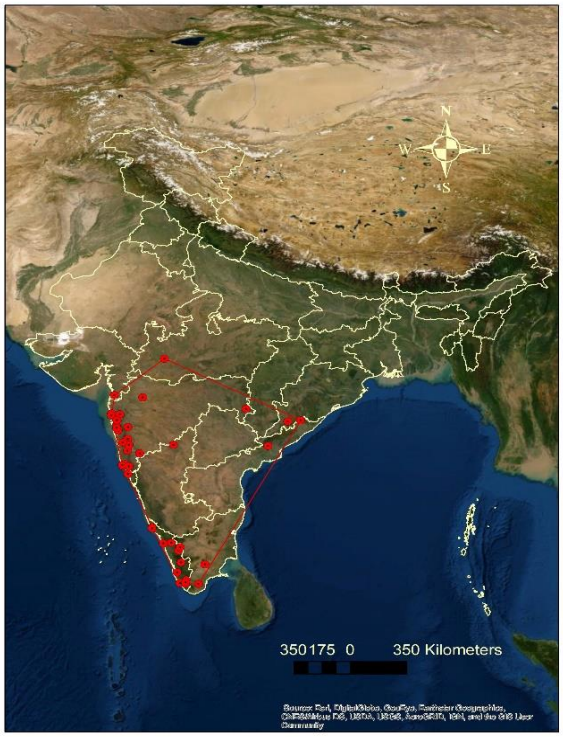
Red list assessment: Critically Endangered [CR B1ab(iii)+2ab(iii); C2a(ii)].

Final assessment in Indian perspective: The assessment is of global perspective as the taxon is endemic. Critically Endangered [CR B1ab(iii)+2ab(iii); C2a(ii)]

Notes: Barbhuiya *et al*. (2017) have treated this as conspecific under *Acampe carinata* **(= *Acampe papillosa*)**. This variety can be recognised by its uniform yellowish colour in sepals and petals which shows consistency in subsequent generation is significant to treat this as a separate variety. The type locality is one of the rich habitats for the variety proper i.e., var. *papillosa* and the variation is within

its population. This variety could also be located in the Duars area during 2016 with similar variations. Thus, it is treated here as a distinct taxon.

3. *Acampe praemorsa* (Roxb.) Blatt. & McCann, J. Bombay Nat. Hist. Soc. 35: 495.1932; Santapau & Kapadia, Orch. Bombay: 233. 1966; A. Abraham and Vatsala, Intr. Orchids: 450, f.135. 1981; R.S. Rao, Fl. Goa Diu Dadra & Nagarhaveli 2: 416. 1986; A.N. Henry & al., Fl. Tamil Nadu 3: 3. 1989; Vajr., Fl. Palghat District, Kerala: 465. 1990; Lakhsmin. in B.D. Sharma & al. (eds.), Fl. Maharashtra: 11.1996; K.N. Ramakrishna in Pull. (eds.), Fl. Andhra Pradesh 3: 93. 1997; S. Misra, Orch. Orissa: 584. 2004; Subba Rao & Kumari in P.S.N. Rao (ed.). in Fl. Visakhapatnam District, Andhra Pradesh 2: 238. 2008; R. Manik. & Lakhsmin., Fl. Rajiv Gandhi



Map 4 Distribution map of *Acampe praemorsa* (Roxb.) Blatt. & McCann

National Park, Karnataka: 377. 2013; Datar & Lakshmin., Fl. Bhagwan Mahavir (Molem) National Park, Goa: 233. 2013; Jalal, Orch. Maharashtra: 36. f. 4.1. 2018; Sushil K. Singh & al., Orch. India: 35. 2019. **Type:** India, Circar mountains, *W. Roxburgh* s.n. (holo. BM). *Epidendrum praemorsum* Roxb., Pl. Coromandel 1: 34. 1795. *Cymbidium praemorsum* (Roxb.) Sw., Nova Acta Regiae Soc. Sci. Upsal. 6: 75. 1799. *Sarcanthus praemorsus* (Roxb.) Lindl. ex Spreng., Syst. Veg. 3: 721. 1826. *Sarcochilus praemorsus* (Roxb.) Spreng., Syst. Veg. 3: 721. 1826. *Vanda congesta* Lindl., Edwards's Bot. Reg. 25: misc. 61. 1839. *Vanda wightiana* Lindl. ex-Wight, Icon. Pl. Ind. Orient. 5: t.1670. 1851. *Saccolabium papillosum* auct. non. Lindl.: Wight, Icon. Pl. Ind. Orient. 5: 9, t.1672. 1851; Dalzell & Gibson, Bombay Fl.: 264. 1861. *Acampe excavata* Lindl., Fol. Orchid. 4 (Acampe): 3. 1853. *Saccolabium praemorsum* (Roxb.) Hook.f., Fl. Brit. India 6: 62. 1890 (excl. syn. *Aerides undulata*). non. (Willd.) Lindl. *nom. illeg.*; Prain, Bengal Plants: 1022. 1903; Haines, Bot. Bihar Orissa 6: 1180. 1924. *Gastrochilus praemorsus* (Roxb.) Kuntze, Revis. Gen. Pl. 2: 661. 1891. *Acampe wightiana* (Lindl. ex-Wight) Lindl. Fol. Orchid. 4 (Acampe): 2. 1853; T. Cooke, Fl. Pres. Bombay 2: 705. 1907; C.E.C. Fisch. in Gamble, Fl. Pres. Madras 3: 1447. 1928. *Vanda fasciata* Gardner ex Lindl. Fol. Orchid. 4

Fig. 4. *Acampe praemorsa* (Roxb.) Blatt. & McCann: A. Habit; B. Capsules; C. Mature capsule with seeds; D. Inflorescence; E. Side view of flower; F. Front view of flower; G-H. Sepals and Petals; I–K. Labellum; L. Front view of lip and column; M. Labellum & ovary; N. Front view of column; O. Side view of column; P-Q. Anther cap; R. Pollinia.

(Acampe): 2. 1853. *Acampe congesta* (Lindl.) Lindl., Fol. Orchid. 4 (Acampe): 2. 1853; C.E.C. Fisch. in Gamble, Fl. Pres. Madras: 1447. 1928; C.S. Kumar & Manilal in Manilal & C.S. Kumar (eds.), Orchid Memories: 163. 2004. **syn. nov.** *Saccolabium wightianum* (Lindl. ex-Wight) Hook.f., Fl. Brit. India 6: 62. 1890, *nom. illeg.*; Gammie, J. Bombay Nat. Hist. Soc. 20: 126, t.10. 1910. *Saccolabium congestum* (Lindl.) Hook.f., Fl. Brit. India 6: 63. 1890. *Gastrochilus congestus* (Lindl.) Kuntze, Revis. Gen. Pl. 2: 661. 1891.

Epiphytic, monopodial herbs up to 70 cm or more. Stem woody, erect to ascending, internodes 1–1.2 cm long, enclosed in persistent, tubular, imbricate, finely striated sheaths. Leaves distichous, coriaceous, 8–20 × 1.5–3 cm, linear-oblong, entire, unequally bilobed at apex, deeply channeled, mid-vein prominent, T.S. of leaf 'V' shaped. Inflorescence lateral, extra-axillary, corymbose, sometimes branched, 3–6 cm long, much shorter than leaves, peduncle with annular sheaths at base, densely flowered. Pedicel and ovary 5–6 mm, glabrous. Flowers 1.2–1.8 cm across, not widely opening, pale yellow with purplish-brown horizontal bands; labellum white, with purple spots at the base; sepals and petals thick, fleshy; dorsal sepal 9–10 × 4.5–5 mm, ovate-oblong, entire, obtuse; lateral sepals 8–9 × 4.5–5 mm, slightly oblique, oblong-ovate, entire, obtuse; petals 7–8 × 1.5–2 mm, oblanceolate, entire, sub-falcate, obtuse. Labellum 6–7 × 4–4.5 mm, fleshy, concave at base, 3- lobed; side lobes small, erect, entire, truncate; mid-lobe broad, deflexed, ovate, erose-undulate, truncate at apex, surface densely pubescent, not warty; spur reduced, sac like, pubescent inside. Column 2–2.5 mm, stout, with 2 erect conical horns at apex; anther cap 2 × 2 mm long, triangular-ovoid; pollinia 4 in unequal pairs, waxy, yellow, globose; stipe 0.5 mm slender; viscidium small, glandular. Capsules 4–5 cm long, cylindrical, longitudinally ridged.

Phenology: March – December.

Habitat: Epiphytic; found in dry deciduous forests and coastal forests from sea level up to 700 m.

Distribution: India (Andhra Pradesh, Chhattisgarh, Daman & Diu, Dadra & Nagar Haveli, Goa, Gujarat, Jharkhand, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Odisha, Rajasthan, Tamil Nadu); Sri Lanka.

Exsicata: Andhra Pradesh: near Gummada, 16.05.1979, *G.V. Subba Rao* 62439 (CAL); Polavaram, Papikanda R.F., 27.09.1962, *D.C. S. Raju* 503 (CAL). **Daman & Diu:** Daman, 04.05.63, *S.R. Rolla* 89008 (CAL). **Goa:** Parvorim village, 04.09.1963, *K.C. Kanodia* 89755(CAL); Molem, 15.02.2003, *Datar* 187592. **Kerala:** Kottayam, Nalukodi, Changanacherry, 20.05.1987, *V.T. Sastry* 1438 (CAL); Kasaragod, Olavara, 12.05.1982, *V.J. Nair* 73864 (CAL); Quilon, Nadayara, 18.12.1979, *C.N. Mohanan* 63794 (CAL). **Madhya Pradesh:** Indore near Haralpur-5miles to Manpur, 24.04.1963, *A.R.K. Sastry* 87617 (CAL). **Maharashtra:** Gadchiroli: Surajgad, *Govekar* 1652 (BAMU); Kolhapur: Udegiri, *M. Sardesai* 1723 (SUK); Amba, *M. Sardesai* 1264 (SUK); Kaas, *M.P. Bachulkar* 5010 (SUK); Mumbai Suburban: Borivali, 19.04.1956, *P.S. Herbert* 1615 (BLAT); Andheri, 03.05.1949, *H. Santapau* 10015 (BLAT); Malad, 30.04.1959, *G.L. Shah* 9846 (BLAT); Goregaon, 13.04.1958, *S.C. Tavakari* 1055 (BLAT); Devbag, Malvan, 20 m, 24.07.2012, *J.S. Jalal* 200504 (BSI); Adale, Vengurla, 5 m, 27.08.2013, *J.S. Jalal* 194988 (BSI); Pulas, Sawantwadi, 65 m, 29.08.2013, *J.S. Jalal* 195028 (BSI); Thane: Mal, 100 m, 24.11.2012, *J.S. Jalal* 200729 (BSI); Kamda forest Badlapur range, 07.06.1967, *K.V. Billore* 110803 (BSI); near Usla village, Murbad range, 13.04.1968, *K.V. Billore* 110903 (BSI); Mumbra, 25.03.1954, *K.V. Shenoy* 2437 (BLAT); Dudhani, 10.05.63, *S.R. Rolla* 89181 (CAL); Ghodbunder, 12.05.75, *N. Subrahmanyam* s.n. (CAL). **Odisha**: Koraput, Kondakamberu, 25.04.63, *D.C. S. Raju* 847 (CAL). **Tamil Nadu**: Travancore, Tenmalai on rock behind Bungalow, 12.09.1918, *C.C. Calder & M.A. Ramaswami* 870 (CAL); Travancore, Dec-10, *A. Meebold* 12957 (CAL); Tirunelveli, Palakkadu, Karaiyar to Gouthalaiyar, 23.05.1988, *R. Gopalan* 88638 (CAL).

Extent of occurrence (EOO): 103747.80 km^2. **Area of Occupancy (AOO):** 488 km^2.

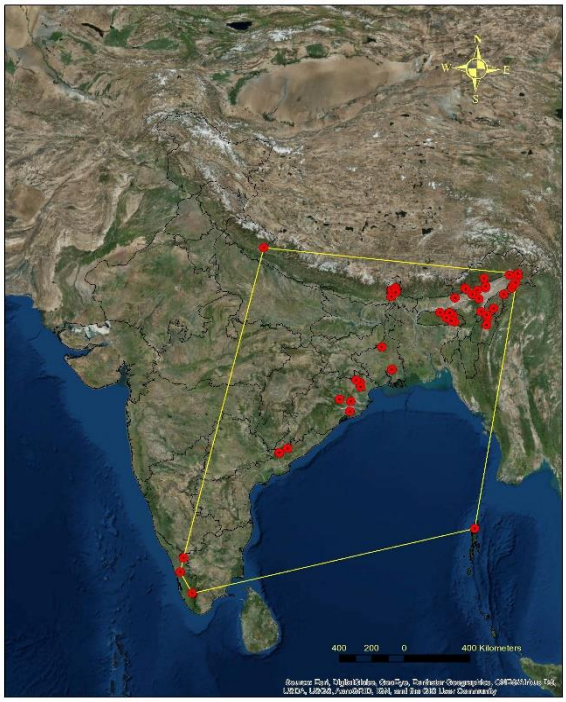
Red list assessment: Endangered [EN B2ab(iii)+C2a(i)].

Final assessment in Indian perspective: Vulnerable° [VU°].

Notes: This species is mis-understood by many authors and often mixed together with either *Acampe papillosa* or *Acampe rigida*. It has vegetative structure, more allied to *Acampe papillosa* whereas the flowers are closer to *Acampe rigida* with very short, shallow spur and densely pubescent, non-warty mid-lobe of labellum. The imperfectly known *Acampe congesta* has been placed herewith because of extreme similarity in both vegetative as well as floral characters.

4. *Acampe rigida* (Buch. -Ham. ex J.E. Sm.) P.F. Hunt, Kew Bull. 24: 98. 1970; Pradhan, Indian Orchids 2: 524. 1979; Deva & H.B. Naithani, Orch. Fl. NW Himalaya: 357, f.203a. 1986; Kataki, Orch. Meghalaya: 182. 1986; Seidenf., Opera Bot. 95: 45, f.24. 1988; Lakhsmin. in B.D. Sharma & al. (eds.), Fl. Maharashtra: 11.1996; H.J. Chowdhery, Orch. Fl. Arunachal Pradesh: 41, f.13. 1998; Hynn. & al. in Hajra & U. Chatterjee (eds.), Orch. Nagaland: 28. 2000; N. Pearce & P.J. Cribb, Orch. Bhutan: 491. 2002; C.S. Kumar & Manilal in Manilal & C.S. Kumar (eds.), Orchids Memories: 163. 2004; S.

Misra, Orch. Orissa: 587, f.588 – 590. 2004; Lucksom, Orch. Sikkim: 806, f. 486, pl. 35. 2007; D.K. Ghosh & J.K. Mallick, Fl. Darjeeling Himalayas: 654. 2014; K. Gogoi, Wild Orch. Assam: 29. 2017; Sushil K. Singh & al., Orch. India: 39. 2019. **Type**: Nepal, icon Buchanan-Hamilton (holo. LINN, photo!). *Aerides rigida* Buch. -Ham. ex J.E. Sm. in Rees, Cyclop. (Addenda) 39: *Aerides*, no. 12. 1819. *Vanda multiflora* Lindl., Coll. Bot.: t.38. 1826. *Vanda longifolia* Lindl., Gen. Sp. Orchid. Pl.: 215. 1833. *Acampe multiflora* (Lindl.) Lindl., Fol. Orch. Acampe: 1. 1853; R.S. Rao, J. Bombay Nat. Hist. Soc.: 322. 1964; H.J. Chowdhery, Orch. Fl. Arunachal Pradesh: 39. 1998. *Acampe longifolia*



Map 5 Distribution map of *Acampe rigida* (Buch.-Ham. ex Sm.) P.F. Hunt

(Lindl.) Lindl., Fol. Orchid. 4 (Acampe): 1. 1853; A.S. Rao & N.P. Balakr., Rec. Bot. Surv. India 20(2): 204. 1973. *Acampe intermedia* Rchb.f. in Otto. & Dietr., Allg. Gartenz 24: 217. 1856; Hook.f. Fl. Brit. India 6: 66. 1890. *Acampe wightiana* var. *longipedunculata* Trimen, Syst. Cat. Ceylon: 90. 1885. *Saccolabium longifolium* (Lindl.) Hook.f., Fl. Brit. India 6: 62. 1890; King & Pantl., Ann. Roy. Bot. Gard. Calcutta 8: 220. t.292. 1898. *Acampe praemorsa* var. *rigida* (Buch. -Ham. ex J.E. Sm.) Barbhuiya, D. Verma & Vik. Kumar, Phytotaxa 303(3): 274. f.3. 2017.

Plants up to 100 cm long or more, often forming large colonies. Stem much stout, elongated, erect or ascending; internodes 2–5 cm long, 10–15 mm diam., enclosed by sheathing leaf-bases. Leaves distichous, all along the stem, 10–45 × 3–5 cm, fleshy, oblong, obtuse, unequally and obliquely 2-lobed, mid-vein thickened below, base articulate, sheathing, lignified and striated at maturity.

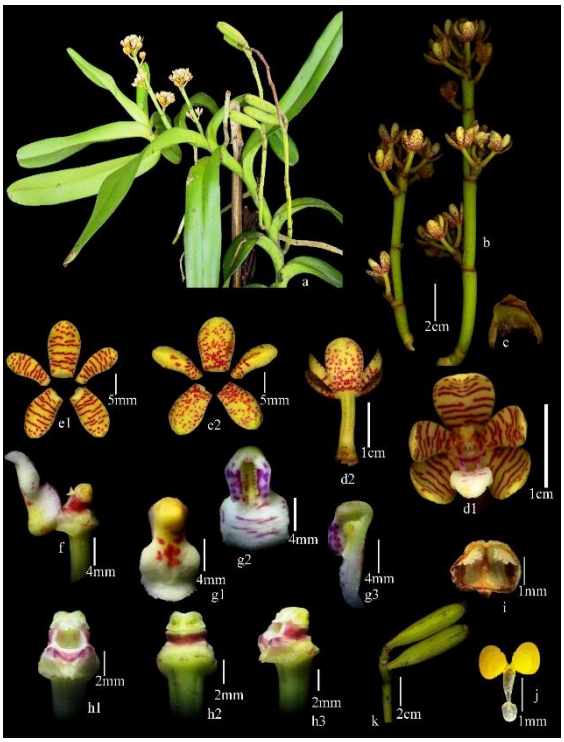


Fig. 5 *Acampe rigida* (Buch.-Ham. ex Sm.) P.F. Hunt: a. Habit; b. Inflorescence; c. Bract; d1 - d2. Flower (front and back view); e1 - e2. Sepals & petals (ventral and dorsal view); f. labellum with column; g1 - g3. Labellum (front, back and side view); h1 - h3. Column (front, back and side view); i. anther cap; j. pollinia; k. capsules.

Inflorescence 1-many, extra-axillary, corymbose, often branched, erect, rigid, much shorter than leaves, 5–20 cm long; peduncle 1–4 cm long (up to 15 cm in branched ones), thick, jointed at nodes, with brownish, annular sheaths; rachis 1–2 cm long, thick, ridges, densely many-flowered. Floral bracts 3–3.5 × 3–3.5 mm, ovate-triangular, obtuse, clasping, greenish with maroon spots, persistent. Flowers spirally arranged, 15–20 mm across, not widely spreading, slightly fragrant, pale yellow with purplish-brown, transverse bands at ventral surface and with purplish-brown spots dorsally; labellum white to pale yellow with pink-purple bands and spots at both surfaces, spur yellowish, column pink-purple in front. Pedicel plus ovary 10–17 mm long, fleshy, greenish-yellow. Sepals and petals thick textured, rigid, weakly concave, dorsally thickened; dorsal sepal 10–14 × 7–9 mm, oblong-obovate, rounded; lateral sepals 9–13 × 6–8 mm, slightly falcate, oblong-obovate, rounded; petals 8–12 × 4–5 mm, oblanceolate-spathulate, falcate, rounded. Labellum 8–10 × 4–5 mm (through mid-lobe), saccate at base, 3-lobed; side-lobes conspicuous, broadly triangular, erect;

mid-lobe large, deflexed at apex, ovate, erose-undulate, obtuse-truncate, upper surface densely glandular-pubescent, not warty; disc with a densely pubescent, elevated, longitudinal callus at centre, extending up to base of mid-lobe; spur highly reduced, represented by a broadly triangular, shallow sac, 1.5–2 × *c*. 2 mm, pubescent inside. Column 2–3 mm tall, stout, with two erect, conical horns at apex; anther sub-orbicular; pollinia globose; stipe *c*. 1.5 mm long, clavate; viscidium oblong, small; rostellum bifid. Capsules 5–7 × 0.6–0.8 cm, erect, cylindrical-clavate, ridged, sub-sessile.

Phenology: April – December.

Habitat: Epiphytic, grows on trees in moist deciduous and semi evergreen forests up to 1500 m.

Distribution: India (Andaman & Nicobar Islands, Andhra Pradesh, Arunachal Pradesh, Assam, Jharkhand, Kerala, Manipur, Meghalaya, Mizoram, Nagaland, Odisha, Sikkim, Tripura, Uttarakhand, West Bengal); Africa; Bangladesh; Bhutan; China; Indo-China; Malaysia; Myanmar; Nepal; Philippines; Sri Lanka; Thailand.

Exsicata: Andaman & Nicobar Islands: North Andamans, HE Project Area, Sep. 2005. *P.G. Diwakar* 17595 (PBL). **Arunachal Pradesh:** Tirap, Chenglang, 16.10.1959, *R.S. Rao* 20238 (ASSAM); Nisa to Niusa, 29.08.1958, *G. Panigrahi* 14847 (ASSAM); Lohit, Tezu to Denning Road 20.09.1969, *A.S. Rao* 47991 (ASSAM). **Assam:** Kadam R.F, 12.03.1962, *G. Panigrahi* 27781 (ASSAM); Lakhimpur, North Lakhimpur, *Baruah* 74019 (ASSAM); Lakhimpur, 26.11.1957, *G. Panigrahi* 11571 (ASSAM, CAL); Udalguri, Rowta, 08.09.1982, *S.N. Hegde* 3663-2 (OHT). **Kerala:** Thrissur, Peechi Range, 04.09.1976, *K. Ramuamurthy* 47626 (CAL). **Manipur:** Saikul, *C. Sathish Kumar* 28750 (TBGT). **Meghalaya:** Khasi and Jaintia Hills, 30.07.1958, *G.K. Deka* 1401 (ASSAM); Umsaw forest, 10.04.1949, *G.K. Deka* 36169 (ASSAM); Nongpoh, 06.11.1952, *G.K Deka* 36171 (ASSAM). **Nagaland:** Kohima, *Hynniewta* 79977; Nyasia, *Hynniewta* 79948, Zunhebato, *Hynniewta* 80835 (ASSAM). **Odisha:** Malkangiri, Balimela to Bandamamidi, 24.05.1959, *G. Panigrahi* 18561 (ASSAM); Mayurbhanj, Simlipal, Jenabila, 18.06.1985, *S. Misra* SM 848 (CAL). **Sikkim:** East Sikkim, Ranipool – 32$^{th\ mile}$ (Namli), 1100 m, 13.06.2014, *D. K. Agrawala* 37851 (BSHC); Dikchu Right- Flank, 800 m., 09.12.1997, *B. K. Shukla* 19682 (BSHC); Tropical Valleys, May & June 1892, *Pantling* 250 (CAL). **Uttarakhand:** Pithoragarh, 20.05.1983, *Hajra* 74471 (BSD). **West Bengal:** Calcutta, *Schiller* s.n. (B); Birbhum, Kotasur (Sainthia), 23.07.1966, *Basak* 451 (CAL); Kurseong, 16.04.09, Kari 1068 (CAL).

Extent of occurrence (EOO): 3291804 km^2

Area of Occupancy (AOO): 212 km^2

Red list assessment: Endangered [EN B2ab(iii); C2a(i)].

Final assessment in Indian perspective: Vulnerable° [VU°].

Notes: *Acampe rigida* (Buch. -Ham. Ex Sm.) P.F. Hunt and *Acampe praemorsa* (Roxb.) Blatt. & McCann are quite close together and often treated as similar species. However, in this study they are found as distinct species (refer key to the species). Whereas, *Acampe rigida* is widespread in most part of the country, *Acampe praemorsa* is confined only in peninsular India.

Conclusion

This study has provided complete taxonomic information on five taxa of Indian Orchids. This will not only help in correct identification of these species, but also help in understanding the relationship with allied taxa. The threat status assessment as per IUCN guidelines will catalyze conservation action and help in sustainable utilization of this important bioresource. The identification standards developed in the study will definitely help the custom authorities to check bio-piracy. These species, together with other Orchidaceae members are included in the APPENDIX-II of CITES. Members of *Acampe* are having good ornamental potential and are compatible in hybridization experiments. Present study will surely help in understanding this important group of plants.

Acknowledgements

The authors are thankful to the Director, Botanical Survey of India, Kolkata, the Head of Office, Botanical Survey of India, Sikkim Himalayan Regional Centre, Gangtok and the Head of the Department of Botany, Raiganj University for facilities and encouragement. The Ministry of Environment, Forests and Climate Change, New Delhi is thankfully acknowledged for financial assistance to the project 'Systematics and conservation of Indian orchids with special emphasis to Himalayan species' under Himalayan Research Fellowship scheme of National Mission on Himalayan Studies. The competent authorities of all Forest Department are thanked for providing permission and necessary help during the field survey**.** I am highly indebted to Dr. Jewan Singh

Jalal (Scientist 'D'), Botanical Survey of India, Kolkata for providing me illustration of *Acampe praemorsa*.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 61-67

Research Article

PhytoResp : A Database for Medicinal Plants of Darjeeling, Against Respiratory Ailments

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Abstract

PhytoResp is a digital, manually curated database developed at the Department of Botany, University of North Bengal (NBU). The database has been created with the help of 200 published research articles and the eleven volumes of 'Chiranjib Banousadhi' series, written by Shibakali Bhattacharya. Here, we provide the names of 329 medicinal plants that are available in the Darjeeling district region of North Bengal and are traditionally used in the treatment of respiratory diseases. The list of reported plants is included, along with their parts used. They are known to cure 38 respiratory diseases. Among these, 113 plants have already been reported as a cure for COVID-19 (in silico or in vivo). Also, we have taken a step to evaluate the efficiency of the phytocompounds through in-silico methods.

Article info

Received **15 December 2022**
Revised **09 May 2023**
Accepted **27 May 2023**

Keywords: Ethnobotanical databases; Medicinal plants; Phytocompounds; Respiratory diseases.

DOI: *https://doi.org/10.55734/nbujps.2022.v14i01.009*

Introduction

In last few years, the world experienced an unprecedented pandemic called COVID-19. According to WHO, the pandemic took 6,850,594 lives and 757,264,511 positive cases were reported worldwide, as of 21st February 2023 (https://covid19.who.int). It has been experienced that the people who had comorbidity, especially respiratory problems, were particularly vulnerable to COVID-19 pandemic (Beltramo et al., 2021).

One of the main causes of respiratory problems is viral infection. Mainly upper respiratory tract infections such as Common cold is a large burden on society, economically and socially. The most common causative viruses are rhinovirus, influenza virus, adenovirus, enterovirus, and respiratory syncytial virus (Thomas and Bomar, 2022). Moreover, in a study conducted by Beltramo et al. (2021) on whether patients with chronic respiratory diseases (CRDs) had a higher risk of COVID-19 than that of patients with influenza, it was concluded that patients with prior respiratory diseases were globally at higher risk of developing severe COVID-19. They also had a higher mortality rate (except for asthma) when compared with patients who had COVID-19 but did not have prior CRDs or influenza. On the other hand, patients who survived COVID-19 may develop respiratory complications ranging from persistent symptoms and radiologically observable changes to impaired respiratory physiology, vascular complications, and pulmonary fibrosis (Al-Jahdhami et al., 2022).

In cases of respiratory viral infection, no effective medicines are available so far except for symptom relief (Thomas and Bomar, 2022). Herbal drugs on the other hand showed promise in giving relief to respiratory ailments and have been recommended in such conditions related to COVID-19 (Pranskuniene et al., 2022). As a result, more and more researchers are tilting towards phytocompounds as a remedy to such problems. Ayurvedic herbal remedies have also been reported as therapeutic adjuvants in the management of COVID-19 (Borse et al., 2021).

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It is in this context, we, at University of North Bengal decided to create a database which will provide valuable information on the plants, as well as their constituent compounds, that are essentially effective against respiratory diseases. North Bengal is a biodiversity hotspot and University of North Bengal plays significant role in conservation of a number of these plants (Chowdhury et al., 2019). Traditionally, several of these locally available plants are used in treatment of various respiratory diseases (Moktan and Rai, 2019; Panda and Thami, 2022).

In our study, we aim to explore the traditional knowledge on medicinal plants found in the Darjeeling district of West Bengal to find a side-effect free solution to post-COVID respiratory complications. Our study brought into focus a list of 329 medicinal plants and their phytocompounds that may have potential in treatment such complication. Based on this knowledge we developed PhytoResp: a digital, manually curated database which has been created with the help from 200 published research articles and the book series Chiranjib Banousadhi (Vol. 1-11) by Shibakali Bhattacharya. In our database, we tried to document as many of these plants as possible.

Materials and Methods

Tools and databases

Google sites: *Google Sites* is a structured wiki and web page creation tool included as part of the free, web-based Google Docs Editors suite offered by Google (https://sites.google.com/).

Google Scholar: Google Scholar is a freely accessible web search engine that indexes the full text or metadata of scholarly literature across an array of publishing formats and disciplines (http://scholar.google.com/).

Dr. Duke's Phytochemical and Ethnobotanical databases: The database facilitate in-depth plant, chemical, bioactivity, and ethnobotany searches using scientific or common names. (https://phytochem.nal.usda.gov/phytochem/search)

Tropicos: Tropicos is an online botanical database containing taxonomic information on plants, mainly from the Neotropical realm. It is maintained by the Missouri Botanical Garden and was established over 25 years ago (https://www.tropicos.org/).

Plant of the World Online: It delivers information on the taxonomy, identification, images, distribution, traits, threat status, molecular phylogenies and uses of vascular plants worldwide. The data are sourced from the Royal Botanic Gardens, Kew as well as its partners and collaborators (https://powo.science.kew.org/).

e-Flora of India: e-Flora of India is an open-access online database of India's plant diversity to document over 18,000 flowering plant species of India. This portal makes the information in the Flora of India volumes published by BSI available in the digital format (https://efloraofindia.com/).

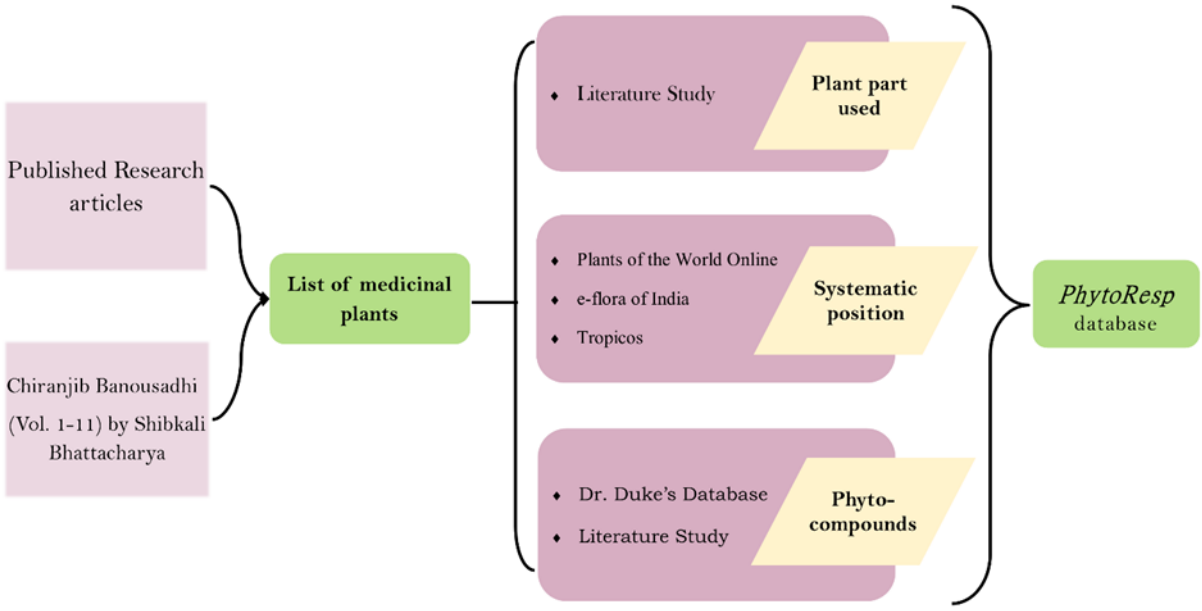

Fig. 1 Schematic overview of the PhytoResp database construction.

Curated list of Traditionally used Medicinal plants in Darjeeling region

The list of plants that are traditionally used against respiratory diseases, and are found in Darjeeling district of West Bengal were compiled through literature study of available research articles from this region and from the book series Chiranjib Bonousadhi (Vol. 1-11) by Shibkali Bhattacharya. Literature search was conducted through *Google sites* with the keywords: respiratory disorders, Darjeeling, cough, cold, fever, traditionally used plants, etc.

After compiling a comprehensive list of all the plants, we mined literature to gather information regarding the various respiratory diseases they are used against and the plant part that is used (Fig. 1).

Taxonomy of the listed plants

The database has a page dedicated to each listed plant that contains the respiratory disease it is used against, the plant part used, the systemic position of the plant, picture and the list of constituent phytocompounds. The databases: Plants of the World Online, e-flora of India and Tropicos were consulted regarding the systemic position and accepted scientific names of the plants. Photographs of the plants were used from Tropicos and Plants of the World Online with proper citation. The data was further validated by Prof. Manoranjan Chowdhury, Department of Botany, University of North Bengal.

Phytochemical composition of the plants

Next the phytochemical constituents of the listed plants were determined. Initially the plants were searched in the Dr. Duke's Phytochemical and Ethnobotanical databases for the list of constituent phytocompounds of each plant. For the plants that were not available in the database, their constituent phytocompounds were determined through literature study. Available research articles were mined through Google scholar and list of the phytocompounds were created manually.

Online Database development

The database has been developed on the *Google Sites* platform. We used the computational languages: CSS, Python and HTML to design the website. The database main page consists of the following interfaces:

PhytoResp: Introduction to our group members who have been instrumental in developing the database.

Home: The homepage provides an idea regarding the significance of our focus on respiratory disorders. It also describes how medicinal plants may have a role in management of respiratory disorders. The page also contains a tutorial on browsing the database.

Browse: Displays an alphabetic list of all the plants that have been studied. Each plant name opens to a page consisting the details and phytocompound list of the plant.

Contact us: Contact information of corresponding author and institute details.

Results and Discussion

Curated list of medicinally used plant

In the beginning of the database construction, we compiled a comprehensive list of 329 medicinal plants of Darjeeling region with the help from 200 published research articles and the 11 volumes of Chiranjib Bonousadhi. These plants belong to 109 different families with the highest number belonging to Fabaceae (23 plants), followed by Asteraceae (13 plants). The top 15 plant families are shown in **Fig. 2**. The plants were reported to be used against 38 respiratory diseases. From the data it was seen that a large number (119) of the plants are being used in treatment of cough (**Fig. 3**). The research articles also reported plants used against bronchitis, tuberculosis, sinusitis, asthma, etc. From our analysis it was also determined that in most cases the administered plant parts are the leaves of the plants (24.5%), followed by root (15.5%) and fruit (14.5%) (**Fig. 4**).

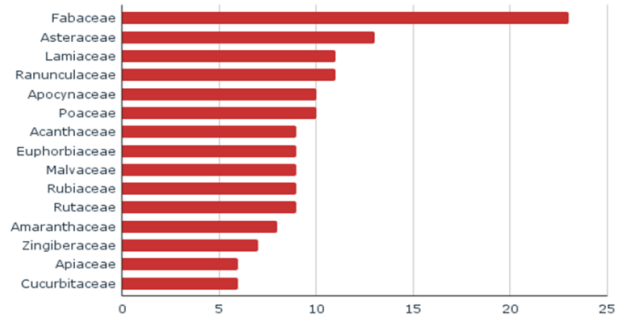

Fig. 2 Top 15 families on the basis of number of plants in our database that belong to them.

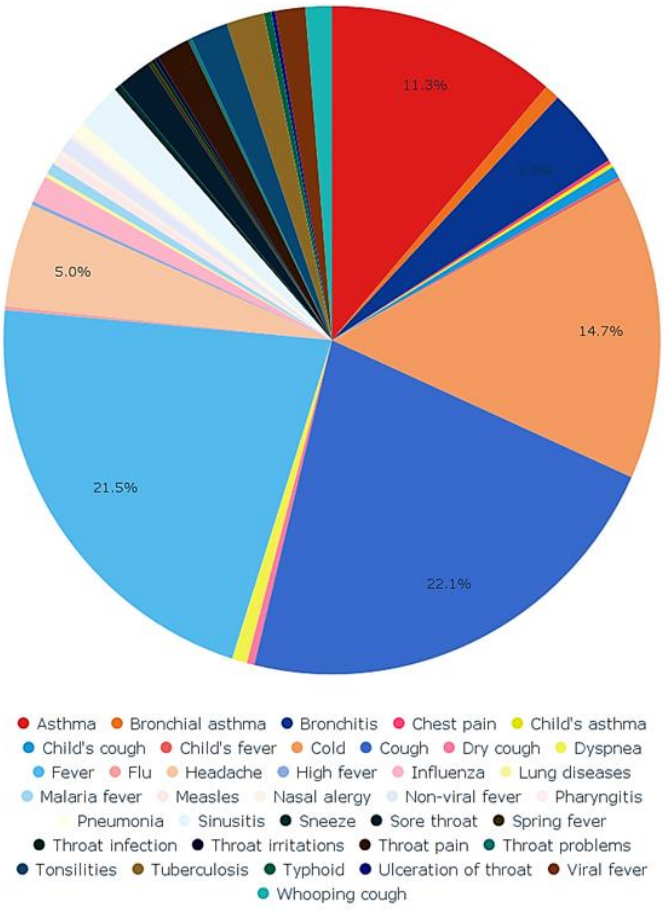

Fig. 3 Chart representing the diseases against which the plants are reported.

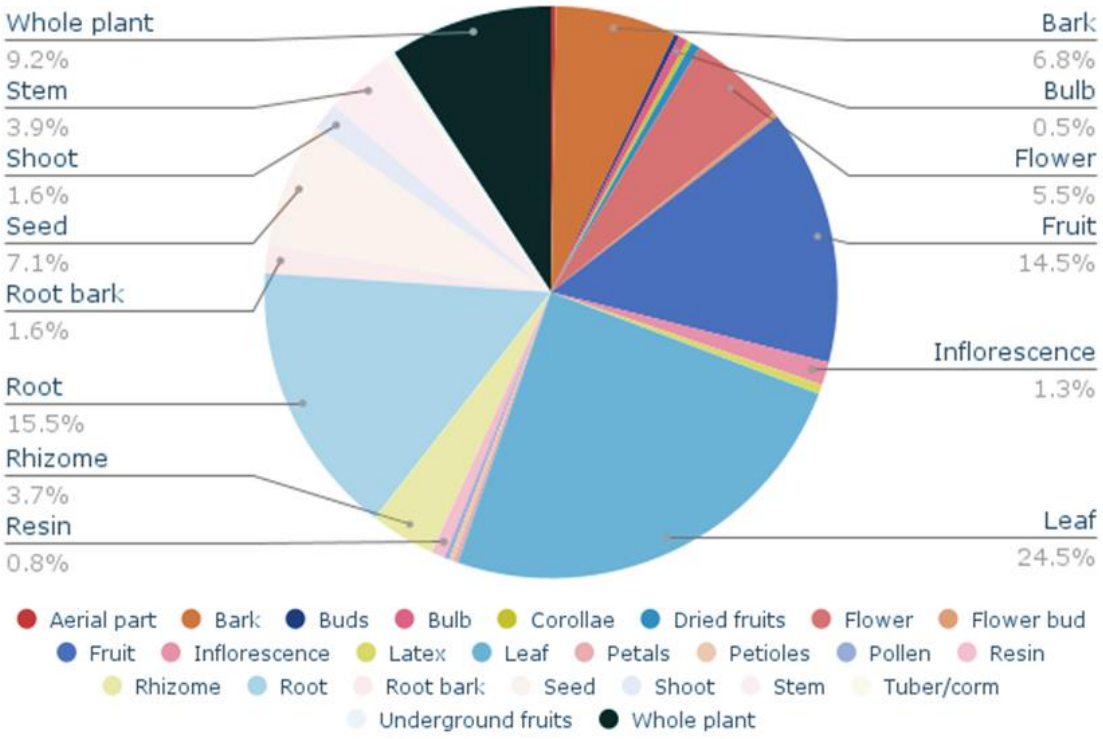

Fig. 4 Chart representing the plant part used in treatment of respiratory diseases.

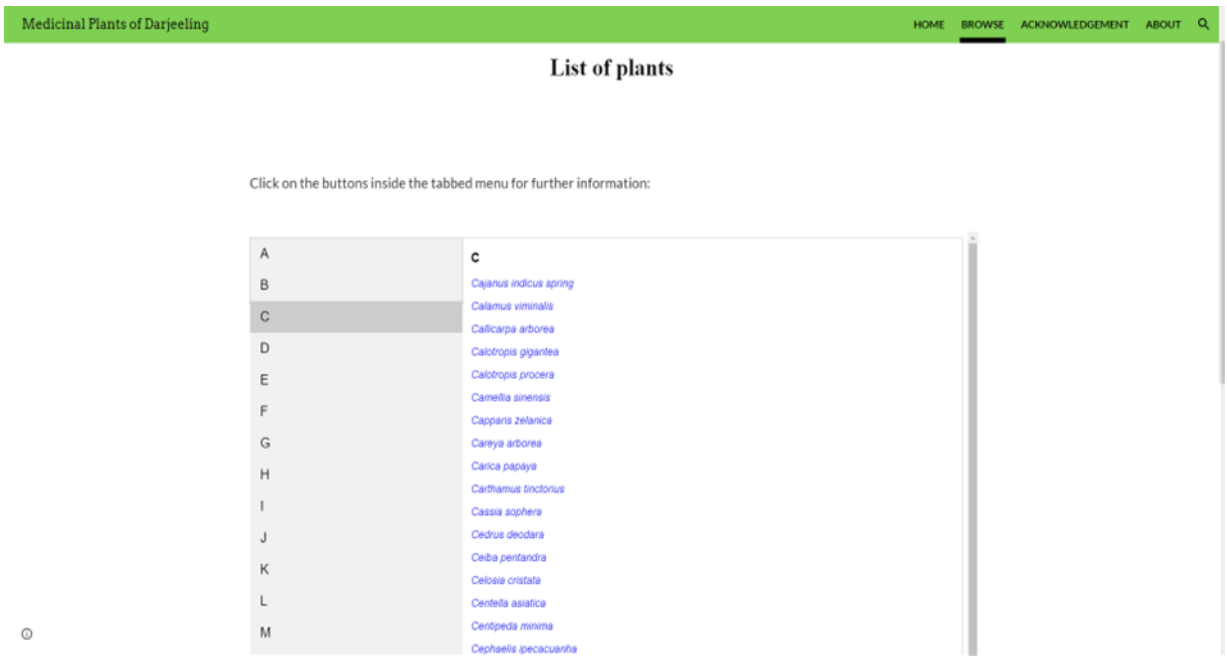

Fig. 5 Snapshot of Browse page of PhytoResp showing list of plants in the database.

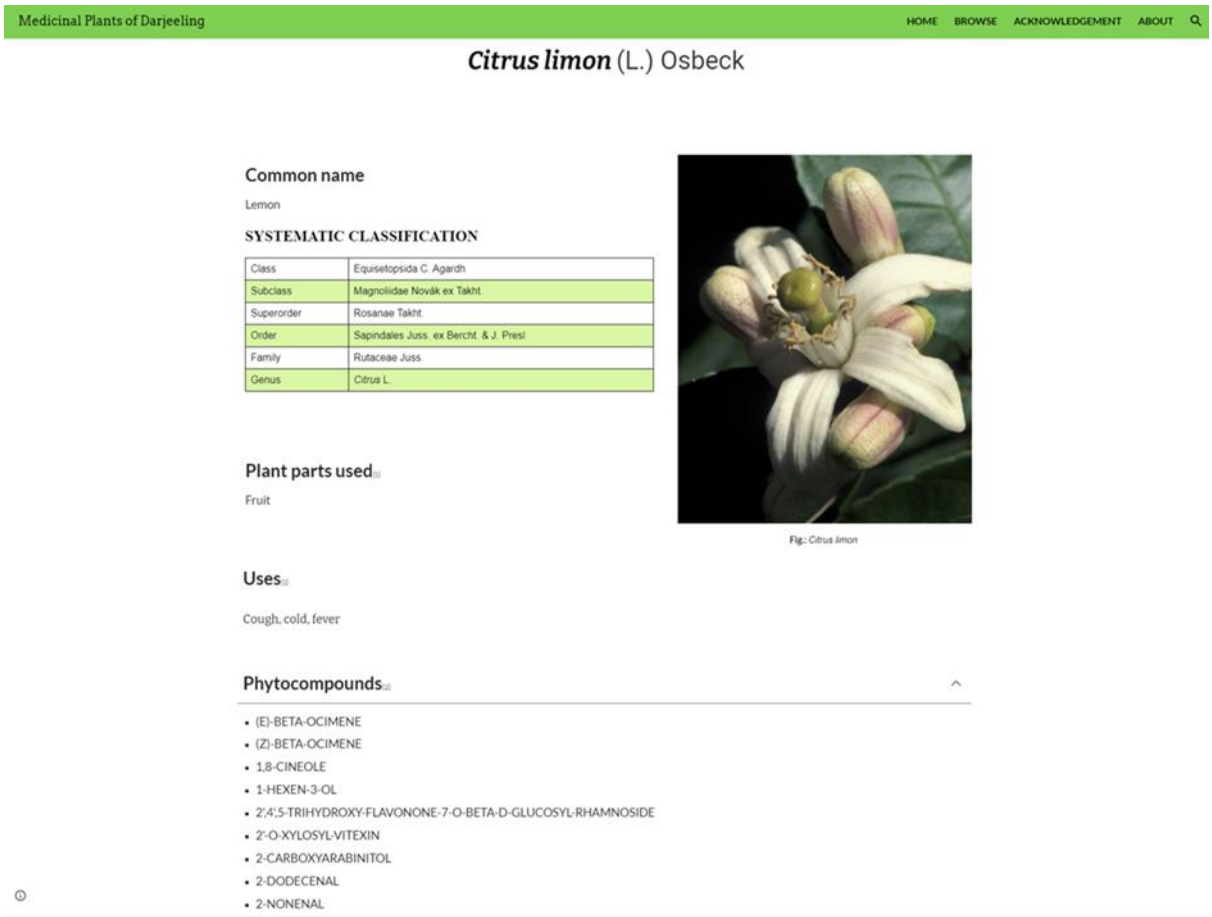

Fig. 6 Snapshot of a page of one of the plants in PhytoResp showing its common name, systematic classification, plant part used, disease treated and list of constituent phytocompounds.

Phytocompounds associated with the plants

The online database constitutes 329 plants, each of which has a webpage dedicated to them (Fig.5). The constituent phytocompounds of all these plants are included in our study. Phytoconstitients of plants have played a role in human health from time immemorial (Dillard and German, 2000). Thus, exploration of the constituents of the studied plants will give an insight into their mechanism of action in treatment of respiratory diseases.

The list of constituent phytocompounds of 108 plants available in Dr. Duke's Phytochemical and Ethnobotanical databases were downloaded. The phytoconstituents of the remaining plants were obtained through literature study. However, articles regarding constituent phytochemicals of some plants were not available.

The available phytocompounds of each plant are listed in the webpage of the respective plants (Fig. 6). The details regarding the phytocompounds will be further added in the database.

Plants in treatment of SARS-CoV-2

Out of the 329 listed plants, 113 are already validated to have therapeutic efficacy against SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), that emerged as a serious human pathogen in late 2019 (Muralidar et al., 2020). The plants were manually searched for their efficacy against SARS-CoV-2. Rest of the traditionally used plants may also have such potential. Thus, proper exploration of the therapeutic efficacy of these plants is needed.

Conclusion

It has been experienced that the people who had chronic respiratory problems were particularly vulnerable to COVID-19 pandemic. Moreover, survivors of the pandemic experienced long-term complications of COVID-19 pneumonia and the main treatment for such respiratory complications is still symptomatic and supportive-care oriented. In this database, we provide the names of 329 medicinal plants found in the Darjeeling district of West Bengal, as well as their parts used, which are known to cure 38 respiratory diseases in Darjeeling region. Among these 329 plants, 113 plants are already reported as a cure to COVID-19 (in silico or in vivo). As a future prospect, we have taken a step to evaluate the efficiency of the remaining plants in treatment of post-COVID respiratory complication through in silico methods.

Acknowledgements

SD acknowledges the University Grants Commission, Government of India for financial support. We acknowledge the Department of Botany, University of North Bengal for allowing us to use the facilities. We would also like to extend our gratitude to Prof. Monoranjan Chowdhury, HOD, Department of Botany, University of North Bengal and Dr. Anirban Biswas, System Manager, Centre for Information and Resource Management, University of North Bengal for their valuable guidance.

Authors' Contribution

AS conceived the idea. AS and SD designed the experiment. AD, AB, and SD executed the study. AD and AB constructed the database. All the authors contributed equally in drafting the manuscript.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 68-74

Research Article

Polyethylene Gycol (PEG) Induced Water Stress in Four Different Genotypes of Pea Seedlings and Evaluation of The Induced Defense Mechanism

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Abstract

The present work was undertaken on artificially induced water stress on pea seedlings under in vitro conditions in order to select the drought tolerant line. Artificial water stress was induced with PEG-6000 on 15-day old seedlings of four varieties for 4, 8 and 12 days. The activities of antioxidative enzymes like peroxidase, catalase, ascorbate peroxidase, glutathione reductase and superoxide dismutase were assayed in the stressed and control plants. POX activity was increased in the initial stages of stress, but its activity was decreased significantly on the 12th day in all the varieties. APOX also showed a similar trend but the maximum activity was noted in Var 3 on the 8th day. CAT activity decreased in var 1 and var 2 when compared with the control, which, however, increased significantly in var 3 and var 4. A slight increase in the GR activity was observed in var 1 and var 2 at the initial stages of the drought stress but its activity decreased significantly on the 12th day in both these varieties when compared with control plants. However, its activity also increased steadily in var 3 and var 4. Maximum SOD activities were noted on the 4th day of drought stress in all four varieties but its activities decreased steadily on the subsequent 8th and 12th days when compared with control. When antioxidative activities were compared among the four varieties, var 3 and var 4 showed maximum increase in antioxidant activity during the period of drought stress. Among the four varieties, var 3 and var 4 showed greater accumulation of H2O2 during the stress days and were maximum at 12th day. Lipid peroxidation also increased in the same varieties. Maximum proline content was noted in both the root and leaf of var 3, followed by var 4. It was further noted that the chlorophyll content decreased significantly in all four varieties in subsequent longer drought stresses. The accumulation of proline content was steadily higher with an increase in the stress length in all the four varieties. During the drought stress, all the varieties showed an increase in ascorbate content but, it was maximum in var 4 followed by var 3 and the least ascorbate was noted in var 1. The present findings indicate that water stress induces oxidative stress in all the four varieties. However, antioxidative mechanisms were found to be more pronounced in var 4 which, therefore, may be considered as the most tolerant to drought stress.

Article info

Received **24 December 2022**
Revised **17 May 2023**
Accepted **28 May 2023**

Keywords: Antioxidant, Abiotic stress, Drought tolerance, Oxidative stress, PEG-6000

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.010***

Introduction

Plants have limited mechanisms of drought stress avoidance; therefore, they require flexible means of adaptation to change drought conditions (Zhang et al., 2004; Pradhan and Chakraborty, 2012). Tolerance to this abiotic stress is a complex phenomenon, comprising a number of physiochemical processes at both cellular and whole organism levels activated at different stages of plant development. Both enzymatic and non-enzymatic antioxidants provide protection against oxidative damage (Munne-Bosch and Algere, 2000). Water induces several physiological and biochemical and molecular responses in several crop plants, which

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would help them to adapt to such limiting environmental conditions (Bajaj et al., 1999; Arora et al., 2002; Lama and Chakraborty, 2012). Drought is a worldwide problem constraining plant production (Chinnusamy et al., 2004) and is prone to acute periods due to little rainfall in the growing season as the environment deteriorates. The lack of adequate moisture leading to water stress is a common occurrence in rain-fed areas, brought about by infrequent rain and poor irrigation (Wang et al., 2005).

Proline and quaternary ammonium compounds, eg. Glycinebetaine, prolinebetaine, etc. are key osmolytes contributing towards osmotic adjustment (Huang et al., 2000; Kavikishore et al., 2005). One of the most important responses of plants to drought and other stress is the overproduction of different types of compatible solutes (Ashraf and Harris, 2004; Serraj and Sincleair, 2002). Of these solutes, proline is widely distributed in plants and normally accumulates in larger quantities than other amino acids in drought-stressed plants (Ashraf, 2004; Irigoyen et al., 1992). Free proline and sugar contents significantly increased in Vigna radiata nodules under drought, but nodules normally contain more praline than leaves (Hooda et al., 1999). Great efforts have been made to decipher the molecular mechanisms of drought tolerance (Bartels and Nelson, 1994; Mahajan and Tuteja, 2005). It inhibits the photosynthesis of plants, causes changes of chlorophyll content and components and damages the photosynthetic apparatus (Escuredo et al., 1998). When plants are subjected to drought stress, a variety of active oxygen species are generated, such as superoxide, H_2O^2 and hydroxyl radicals, which cause damage in plants. They are toxic to living organisms and, unless removed rapidly, they destroy or inactivate various cellular components (Trippi et al., 1989).

Pea (*Pisum sativum*) is an important edible leguminous seed crop for human nutrition. Its seeds contain 18-20% dry matter, and 10-12% is carbohydrate and 5-8% is protein (Vural et al., 2000). Pea is used as a fresh vegetable, frozen or canned. According to FAO 2004 data, about 12.2 million tonnes of pea production were achieved in 6.3 million ha agricultural lands of the world with an average yield of 1.930 kg ha-1 (Anonymous, 2007). In Turkey, the pea production area was 1.568 ha with a total production of 4373 tones and an average yield of 2.79 kg ha-1 in 2006 (TUIK, 2007).

The pea is a cool-season vegetable crop of mild climate regions. Therefore, it gives a higher yield in cold-humid regions compared to warm-dry areas. Its minimum temperature range for germination is between 1-6°C and it can survive in low temperatures up to -5°C. Even though pea can grow in many soils, the best yield can be obtained in clay-loam, deep, productive, moist, slightly acid (pH 6.5-7.0) soils. When the soil is productive and moist, vegetative growth is advanced. On the other hand, pea seed yield decreases. In addition, owing to its taproots, the pea can use a plant's nutrients and water from different soil layers and increase the organic matter content of soil. As a legume crop, the pea is able to fixate 50-150 kg ha-1 nitrogen from air (Sehirali, 1988; Akdag, 2001). Thus, considering the importance of pea cultivation in the district of Darjeeling, the present study was undertaken to determine how four varieties respond to water stress in terms of over expression of antioxidant enzymes or other biomolecules.

Materials and methods

Induction of water stress

Four pea varieties were selected (Table 1) for this present experiment. For induction of water stress, initially seeds were soaked overnight and surface sterilized inside the laminar hood with 0.1% mercuric chloride ($HgCl_2$) (Hi-Media Pvt. Ltd, India) for two minutes and they were rinsed several times with sterile double distilled water to remove $HgCl_2$. The seeds were transferred to sterile petriplates containing sterile double distilled water. The seeds were kept in petriplates and grown in vitro aseptically for 15 days. After 15 days, the seedlings were subjected to drought stress by application of Polyethylene glycol (PEG) 6000 (Hi-Media, Pvt. Ltd, India) and various biochemical tests were performed on 0, 4th, 8th and 12 days of stress along with the control plants.

Table. 1 Varietal names and their respective code names

Sl. No.	Varietal name	Code
1	Palam Priya	Var 1
2	Arka Ajit	Var 2
3	Arka Karthik	Var 3
4	DPPM-65	Var 4

Preparation of the enzyme extract

For determination of enzyme activities, around 1 g of the leaves collected from the treated and control plants were ground to fine powder with a mortar and pestle under liquid nitrogen in 10 ml of cold 50mM sodium phosphate buffer, pH 7.5, containing 1% (w/v) polyvinylpolypyrrolidone (PVP). The

homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was directly used as a crude extract for enzyme assays.

Assay of activities

Peroxidase (POD: EC. 1.11.17)

Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisdin in the presence of H_2O_2 (Chakraborty et al., 1993). Specific activity was expressed as ΔA_{460} mg protein^{-1} min^{-1}.

Ascorbate peroxidase (APX: EC.1.11.1.11)

Activity of ascorbate peroxidase was assessed following the protocol of Nakano and Asada (1981) where its activity was determined by monitoring the decrease in absorbance at 290 nm. The enzyme activity was expressed as ΔA_{290} mg protein^{-1} min^{-1}.

Catalase (CAT: EC. 1.11.1.6)

Catalase activity was estimated by Upadhyaya and Panda (2004). It was calculated by estimating the breakdown of H_2O_2, which was measured at 240 nm. The enzyme activity was expressed as Δ A245 mg protein^{-1} min^{-1}.

Superoxide dismutase (SOD: EC. 1.15.1.1)

The enzyme activity was estimated by monitoring the inhibition of the photochemical reduction of NBT according to the method described by Dhindsa et al., (1981) with some minor modifications. The absorbance of samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzyme.

Glutathione reductase

Glutathione reductase activity was determined by following the protocol of Lee and Lee (2000). It was determined by the oxidation of NADPH at 340 nm. Enzyme activity was expressed as µM NADPH oxidized mg protein^{-1} min^{-1}.

Protein content

Protein content was determined by following Lowry's method (1951).

Lipid peroxidation

Lipid peroxidation was measured following the procedure of Heath and Packer, (1968) where MDA was determined by the thiobarbituric acid (TBA) reaction. The absorbance was measured at 532 nm and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1}.

Estimation of H_2O_2

H_2O_2 was extracted and quantified according to the method described by Jena and Chowdhuri (1981).

Ascorbate

Ascorbate was extracted and estimated by following the protocol of Mukherjee and Choudhuri (1983).

Determination of chlorophyll content

Total chlorophyll was estimated following the standard protocol (Harborne, 1973). Chlorophyll was extracted in 80% acetone and the extract was filtered. Absorbance of the filtrate was noted at 663 nm and 645 nm wavelengths and the chlorophyll content was calculated using a standard formula.

Statistical analysis

Experiments were set up in a completely randomized block design. Each experiment was carried out with 3 replicates. Data was analyzed by one-way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test at p\0.05 (Duncan 1955) on the statistical package of SPSS 10.

Result and Discussion

Four varieties of 15-day-old pea seedlings were subjected to drought stress in vitro by the application of PEG-6000. On the 4th, 8th and 12th days of drought stress plants were sampled for various biochemical assays along with the controls. No significant morphological changes were seen in the experimental plants during the initial stages of drought stress. However, slight wilting of leaves was observed on the 12th day of stress. An essays of antioxidative enzyme activities showed that the POX activity increased in the initial stages of stress (Fig 1a), but its activity was decreased significantly on the 12th day in all the varieties. APOX (Fig 1b) also showed a similar trend but the maximum activity was noted in Var 3 on the 8th day. CAT activity (Fig 1c) decreased in var 1 and var 2 when compared with

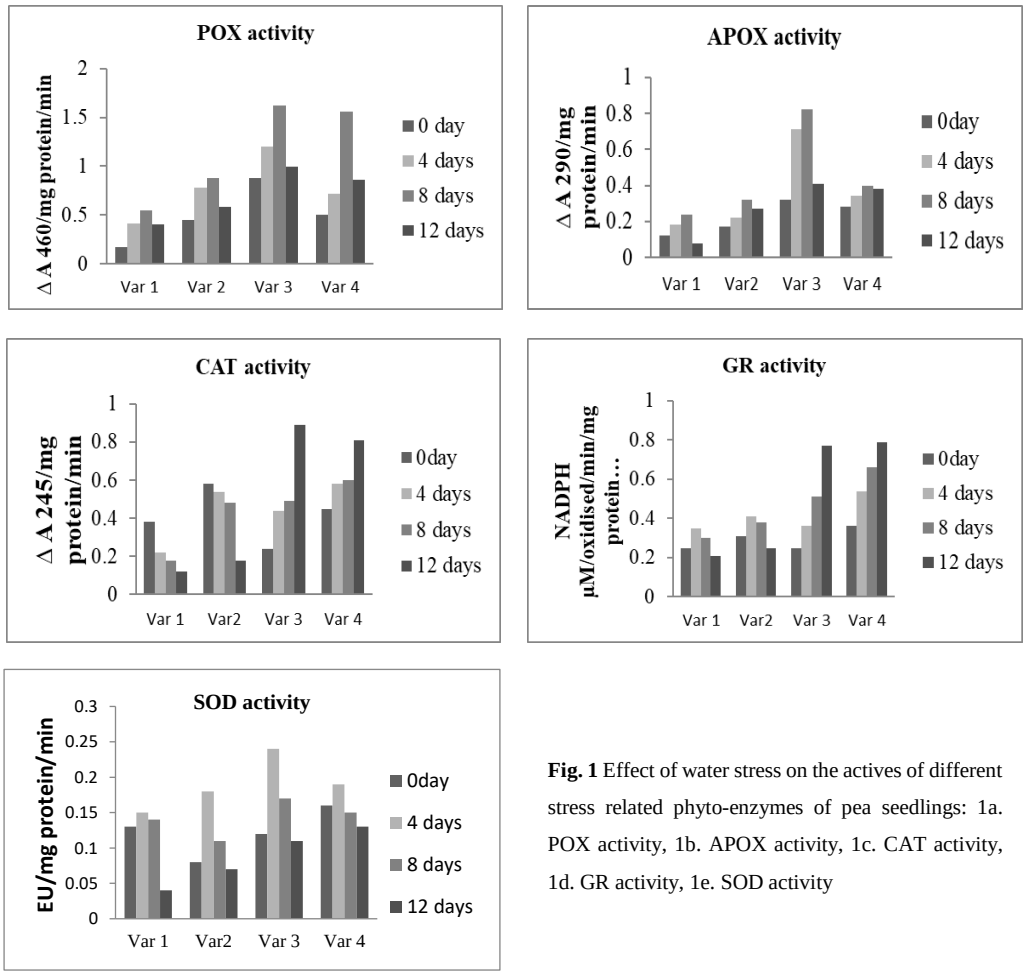

Fig. 1 Effect of water stress on the actives of different stress related phyto-enzymes of pea seedlings: 1a. POX activity, 1b. APOX activity, 1c. CAT activity, 1d. GR activity, 1e. SOD activity

the control, which, however, increased significantly in var 3 and var 4. Similar trends were noted in maize (Lama and Chakraborty, 2012). A slight increase in the GR activity was observed in var 1 and var2 at the initial stages of the drought stress but its activity decreased significantly on the 12th day in both these varieties when compared with the control plants. However, its activity increased steadily even in var 3 and var 4 (Fig 1d). Maximum SOD activities (Fig 1e) were noted on the 4th day of drought stress in all four varieties but its activities decreased steadily on the subsequent 8th and 12th days when compared with control. When antioxidative activities were compared among the four varieties, var 3 and var 4 showed maximum increase in antioxidant activity during the period of drought stress.

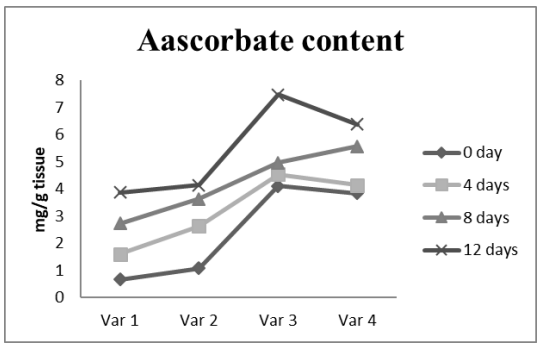

Fig. 2 Effect of water stress on Ascorbate content in pea seedlings

Table. 3 Proline content in leaf and root of four varieties of pea seedlings following water stress.

Varieties	Stress period (d)	Proline content	
		Leaf	*Root*
Var 1	0	0.07	0.19
	4	0.24	0.3
	8	0.42	0.5
	12	0.88	1.11
Var 2	0	0.19	0.3
	4	0.34	0.44
	8	0.66	0.75
	12	0.89	0.98
Var 3	0	0.71	0.98
	4	1.11	1.56
	8	1.98	2.22
	12	2.54	2.75
Var 4	0	0.66	1.11
	4	0.98	1.21
	8	1.45	1.63
	12	**2.45**	**2.11**

capacity of better protective mechanisms against oxidative damage. Among the four varieties, var 3 and var 4 showed greater accumulation of H_2O_2 It has been pointed out by many earlier workers (e.g. Sharma and Dubey, 2005; Tu´rkhan et al., 2005; Lama and Chakraborty, 2012) that maintaining a high level of antioxidative enzyme activities may contribute to drought tolerance by increasing the during the stress days and were maximum on the 12th day (**Table 2**). Lipid peroxidation also increased in the same varieties (**Table 2**).

Table. 2 H_2O_2 accumulation and lipid peroxidation in pea seedlings following water stress.

Varieties	Stress period (d)	H_2O_2 a	Lipid b
Var 1	0	1.55	0.009
	4	5.68	0.018
	8	8.69	0.028
	12	14.69	0.038
Var 2	0	2.22	0.005
	4	6.59	0.017
	8	9.45	0.022
	12	14.11	0.025
Var 3	0	2.05	0.008
	4	7.89	0.012
	8	11.44	0.024
	12	**15.9**	**0.035**
Var 4	0	1.89	0.016
	4	7.88	0.029
	8	12.77	**0.039**
	12	**18.51**	0.021

It was further noted that the chlorophyll content decreased significantly in all four varieties in subsequent longer drought stresses (**Fig. 3**). Similar observations were also made in maize (Lama and Chakraborty, 2012) and barley (Kuroda et al., 1990). The accumulation of proline content was steadily During the drought stress, all the varieties showed an increase in ascorbate content but, it was maximum in var 4 followed by var 3 and the least ascorbate was noted in var 1 (**Fig 2**). Ascorbate can directly act as a free radical scavenger (Bowler et al., 1992; Larson, 1998; Lama and Chakarborty, 2012).

higher with an increase in the stress length in all the four varieties (**Table 3**). Maximum proline content was noted in both the root and leaf of var 3, followed by var 4. Accumulation of proline in plants under stress is a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes and repressed activity of proline degradation (Delauney et al., 1993; Peng et al., 1996). Accumulation of proline is an important indicator of drought in plants including bacteria and algae (Lama and Charaborty, 2012). This important amino acid has been reported to play multiple physiological functions in plants subjected to drought (Lama and Charaborty, 2012).

Conclusion

The current data show that water stress causes oxidative damage in all four varieties of pea. However, antioxidative mechanisms were found to be more evident in var 4, which may be considered the most tolerant to drought. It is generally understood that laboratory circumstances may not always reflect the genuine behaviour of plants subjected to water stress in the field. On the other hand, such findings may aid in understanding the mechanism of drought stress management and the selection or development of drought-resistant pea genotypes.

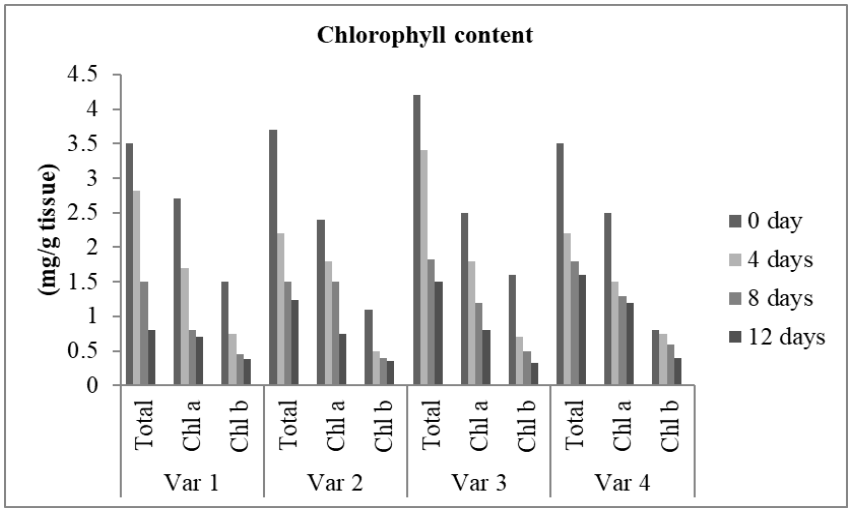

Fig. 3 Effect of water stress on chlorophyll content in pea seedlings

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NBUJPS
NBU Journal of Plant Sciences

ISSN No. 0974-6927
Vol. 14 (2022), pp. 75-83

Research Article

Antifungal Efficacy of Cu-Based Nano-Chitosan on *Rhizopus stolonifer*, A Virulent Phytopathogen

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Abstract

Agro-scientists are giving endless efforts for synthesizing a bio-derived molecule that can act as a promising antifungal agent for combating a large number of phytopathogens. Harmful phytopathogens decrease crop yield and its quality. *Rhizopus stolonifer* is one such virulent phytopathogen that causes huge losses during the post-harvest period of crops. This pathogen mainly causes rot disease in fruits, crops, and vegetables. The second most abundantly available biological macromolecule, Chitosan and its metal-based nanoparticles stands as a potential antifungal agent for combating *Rhizopus stolonifer*. This study includes the synthesis of Cu chitosan nanoparticles (Cu-CNPs) and chitosan nanoparticles (CNPs) through the ionic gelation method and its characterization based on UV Vis spectrophotometer, FE-SEM, EDXS, and DLS. Cu-CNPs and CNPs were screened from 100-2000 µg/mL concentration against *R. stolonifer* for the assessment of its antifungal activity. Spore viability assay and lipid peroxidation of the pathogen using Cu-CNPs and CNPs were also determined. Generation of oxidative stress in the mycelium of the pathogen on the application of Cu-CNPs and CNPs was traced by fluorescence microscopy. Changes in the ultra-structure of the sporangium of *R. stolonifer* after treatment with Cu-CNPs and CNPs were visualized under SEM. Results showed that Cu-CNPs inhibit the growth of *R. stolonifer* at 2000 µg/mL and elevate malonaldehyde (MDA) content in the pathogen as a result of lipid peroxidation and produces defined damages on the sporangium membrane as observed under electron microscope. Fluorescence microscopy revealed the emission of high intensity of fluorescence due to the generation of oxidative stress in Cu-CNPs treated fungal mycelium.

Article info

Received **20 January 2022**
Revised **01 June 2023**
Accepted **08 June 2023**

Keywords: Chitosan, Cu Nanoparticle, Lipid Peroxidation, Phytopathogen, *Rhizopus stolonifer*

DOI: ***https://doi.org/10.55734/nbujps.2022.v14i01.011***

Introduction

Rhizopus stolonifer, a member of the phylum Zygomycota, commonly traced in tropical and subtropical zone, is one of the most virulent pathogens of crops and vegetables (Omoifo, 2011). This pathogen has a worldwide distribution and is mostly found in moldy materials. It subsists in both soil and air. It acts as a major parasite of plant tissue by causing rot disease (Baggio et al., 2015). This pathogen grows vigorously and spreads easily by means of a stolon. The stolon helps the fungus to grow vertically and horizontally (Estrada et al., 2019). They possess sporangiophores of about 2.5 mm long and 20 µm in diameter (Abe et al., 2010). *Rhizopus* causes huge loss of harvested crops during storage, transit, and while marketing. Usually, this pathogen occurs as a saprophyte or often as a facultative parasite in the stored plant products. This pathogen invades plant tissue through infection peg and emerges out from the wounds by producing aerial mycelium, sporangia, sporangiophore, stolon, and rhizoids (Diego, 1997). According to a field

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study by Holeta Agricultural Research Station, field crops suffered a loss of 32-52% due to the infection caused by *Rhizopus stolonifer*. Loss of industrial crops stands between 22 to 44% while horticultural crops showed a reduction in the yield of 35 to 62% (Shtienberg, 1997). *Rhizopus* infects vegetables, ornamental crops, fruits, seeds, grains and nuts. This pathogen majorly causes rot in sweet potatoes, sunflower, strawberry, cherries, peanuts, cucurbits and peaches. Under high level of moisture, they infect cereals and corns. Rhizomes of flower crops, bulbs and corms are also susceptible to this pathogen. Not only in plants but humans are also victimised through mucormycosis caused by *Rhizopus* spp. (Agrios, 2005). *Rhizopus* showed rapid growth and sporulate vigorously under humid conditions with mild temperature. It grows rapidly between 15 to 30ºC an optimal developmental temperature of 23 to 28ºC. The infected areas are characterized by soft watery mass covered with white hairy mycelia and black sporangiophores erected vertically (Agrios, 1997). Due to the comprehensive range of hosts of *Rhizopus stolonifer* and its fast colonization capability, it is important to target its control.

Researchers have proposed various strategies for controlling *Rhizopus stolonifer*. Strategies like crop rotation, the use of synthetic fungicides, and spraying of botanicals in the crops and foliage are age-old management practices of the farmers. Very recently, agro-researchers are trying to derive a potential biomolecule for controlling a broad spectrum of phytopathogens. In this context, nanotechnology has opened up new avenues for the management of crop diseases. Nanoparticles derived from a biological macromolecule- chitosan and its metal derivatives are known to inhibit a maximum number of fungal pathogens (Chouhan et al., 2022). CU nanoparticles (Cu-NPs) hold worldwide attention due to their broad array of antimicrobial properties. It was prescribed by Hippocrates in 400 BC for pulmonary disorders. For centuries CU is known to use for the purification of water. CU is bio-physically active owing to its mechanical, optical, electrical, thermal and catalytical property (Lee et al., 2009; Shende et al., 2015; Umer et al., 2014). CU and its nanoparticles hold strong reviews for having significant bioactivity against bacteria, fungi, viruses and nematodes (Ingle and Rai, 2016).

Chitosan is a biodegradable, biocompatible, non-toxic antifungal agent that provides greater permeability into the fungal membrane. Nanoparticles formed from chitosan are likely to be more permeable into the fungal membrane. It is already reported that chitosan is a promising antifungal agent due to its polycationic nature (Hans and Lowman, 2002). Owing to its polycationic nature, chitosan binds with the negatively charged components of the fungal membrane resulting in a disintegrated structure. Chitosan nanoparticles (CNPs) ensure greater encapsulation capacity with increased surface area (Sarkar and Acharya, 2020). Similarly, attempts have been made the synthesis of metal-conjugated nanochitosan for gain increment in the antifungal activity (Malerba and Cerana, 2016). Metal-conjugated nanoparticles such as (Cu-CNPs) are biologically more active due to their varied structural and functional properties. Metallic nanoparticles are known to generate ROS in pathogens. They cause protein and nucleic acid leakage of the pathogen, followed by hindering the membrane stability (Sathiyabama and Charles, 2015; Sathiyabama and Parthasarathy, 2016; Kong et al., 2010). Therefore, the present paper aims to delineate the hypothesis that Cu when incorporated in CNPs will enhance antifungal activity against *Rhizopus stolonifer*. Concomitantly, on application of Cu-CNPs there will be generation of oxidative stress and peroxidation of fungal lipid that will restrict its growth.

Materials and methods

Synthesis of CNPs and Cu-CNPs

Both the nanoparticles (CNPs and Cu-CNPs**)** were synthesized by following Ionic Gelation Method. Low molecular weight chitosan (80% N-deacetylation; 50,000-190,000 Da) was solubilized in 1% acetic acid solution (v/v) and continuously stirred for 30 mins. 40 mL of Sodium Tripolyphosphate (STPP) was added drop-wise into the chitosan solution with constant stirring. A milky white suspension is formed after 1 hr of further stirring. This solution is used as chitosan nanoparticles (CNPs) solution and stored at 4ºC for further use (Dananjaya et al., 2017a).

Cu-CNPs was prepared by the drop-wise addition of 10 mL of 4% $CuSO_4.5H_2O$ into low molecular weight chitosan solution made in 1% acetic acid solution. The solution was stirred for 20 mins and refluxed at 120ºC. 0.05 M ascorbic acid (0.5 mL) followed by 0.6 M NaOH was (2 mL) added into the solution and stirred for 15 mins. After further stirring, a light green colouration was observed. A quick brown colour appeared following the addition of 0.5 mL N_2H_4. Finally, STPP was added as mentioned above. This solution was centrifuged at 12,000 rpm for 10 mins and supernatant was discarded while the pellet obtained was resuspended in in same amount of distilled

water and used as nanoparticle solution (Usman et al., 2012).

Characterisation of CNPs and Cu-CNPs

UV-Vis (Aligent Technologies, Carry 100 UV-Vis) spectral absorbance of CNPs and Cu-CNPs were obtained by scanning the nanoparticle solutions in the range of 200-800 nm wavelength. The surface morphology of both the nanoparticles were scanned in JSM-7900F Schottky Field Emission Scanning Electron Microscope (FE-SEM); JEOL, with an accelerating voltage of 0.1-30 kV. The existence of elements in Cu-CNPs down to boron were scanned through EDXS analysis. EXDS was particularly performed to confirm the presence of CU in the synthesized nanoparticle. The measurement of hydrodynamic particle size and its distribution were determined through Dynamic Light Scattering (DLS).

Poisoned Food Assay

The effect of CNPs and Cu-CNPs on mycelial growth of *Rhizopus stolonifer* was determined through a poisoned food assay. A series of concentrations (100, 500, 1000, 1500 and 2000 µg/mL) of both nanoparticles were prepared in the form of solution. Nanoparticle solutions were sterilized and mixed with 20 mL of autoclaved Potato Dextrose Agar (PDA) media by maintaining the final required concentration of the nanoparticles in the Petri plates. The experiment was carried out in sterilized petri plates of 90 mm diameter. A 5 mm diameter of mycelial disc was excised from 7 day old culture and placed in the center of the nanoparticles treated plates. A control plate was prepared without the treatment of nanoparticles. Plates were incubated at 28-30ºC and monitored for days until the control plate showed full radial mycelial growth. Three replicates were for each plate. Fungal growth inhibition was measured by using the following formula and it is expressed as percent inhibition of radial growth (PIRG) (Dananjaya et al., 2017a):

$$\text{PIRG (\%)} = \frac{RGC_{Control\ plate} - RGC_{treated\ plate}}{RGC_{Control\ plate}} \times 100$$

Where, RGC – denotes radial growth of fungal colony

Spore viability assay

The viability of the *R. stolonifer* spore was estimated quantitatively by XTT 2,3- Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide. An electron coupling agent Menadione was used in this assay. Spore suspension containing 1×10^6 conidia/mL was prepared in a Czapeck liquid medium. Spore suspension of 100 µL was cultured in 96 well flat bottom microplates for 6 hrs at 28-30ºC. Following incubation, equal amounts of both nanoparticles under different concentrations ranging from 100-2000 µg/mL were added to the suspension. The suspension was further incubated for 4 hrs. 50 µL XTT solution mixed with 7 µL of 25 µM menadione was added to the resulting suspension. Optical density was measured at 450 nm after 3 hrs of incubation at 28-30ºC (Alcaraz et al., 2016).

Determination of Lipid Peroxidation of R. stolonifer

Lipid peroxidation of *Rhizopus stolonifer* after the application of CNPs and Cu-CNPs, was quantitatively measured by malonaldehyde (MDA) estimation. MDA is the indicator of lipid peroxidation. The pathogen was treated with aforesaid concentrations of both the nanoparticles and cultured in potato dextrose broth (PDB) for 7 days at 28ºC. After incubation the harvested mycelial mat when thoroughly washed with sterile distilled water and dried with sterile blotting paper. One gram of mycelial mat was homogenized in 10 mL of chilled 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 10 mins. The pellet was discarded and the supernatant was used for MDA estimation. 100 µL of the mycelial homogenate was mixed with 0.335% (w/v) thiobarbituric acid and 10% of trichloroacetic acid in equal volume. The reaction mixture was boiled in a water bath for 10-15 mins. The absorbance of the color formed in the reaction mixture was measured spectrophotometrically at 530 nm using molar absorption co-efficient 1.56×10^5 (Subban et al., 2019).

Analysis of CNPs and Cu-CNPs on oxidative stress production upon treatment on R. stolonifer

In order to check the generation of oxidative stress in the fungal hyphae a non-fluorescent probe molecule, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) was used. This molecule is non polar hydrophobic in nature and it is easily permeable to the fungal cell. The intracellular esterase present inside the cell results in the deacetylation of the probe into 2',7'-dichlorodihydrofluorescein (DCFH). This deacetylated product gets oxidized by reactive oxygen species produced in the cell as a result of nanoparticle treatment. The oxidized product emits green fluorescence from the fungal hyphae when observed under a fluorescence microscope. The fungal hyphae were treated with the previously

mentioned range of concentrations of both the nanoparticles for 3 days, incubated at 28ºC in PDB. After incubation, the cultural were centrifuged at 10,000 rpm for 12 minutes to obtain the hyphal cells. The hyphal cells were washed with phosphate buffer saline (PBS) 3 times. The hyphae remained suspended in PBS. In a separate microcentrifuge tube, 40 µL of hyphal suspension was mixed with an equal volume of non-fluorescent probe dissolved in methanol under dark conditions. The tubes were incubated for 2 hours in the dark at 28ºC with frequent shaking. The generation of oxidative stress in the cells was observed under a fluorescence microscope with excitation filter of 485 nm and an emission filter of 525 nm (Chen et al., 2020).

Analysis of ultrastructural changes on sporangium of R. stolonifer on CNPs and Cu-CNPs treatment

The changes in the ultra-structural level on the sporangium of *R. stolonifer* were observed under Scanning Electron Microscopy (SEM) after the application of both nanoparticles at 2000 µg/mL concentration. Sporulated fungal cultures produced in PDB were treated with the nanoparticles and incubated for 48 hrs at 28-30ºC. The fungal tissue was pre-treated with glutaraldehyde and washed with graded alcohol before examining under microscope (Dananjaya et al., 2017b).

Results and Discussion

Characterisation of CNPs and Cu-CNPs

The synthesized nanoparticle solutions were scanned in the range of 200-800 nm in a UV-Visible spectrophotometer for the assessment of their optical properties using water as a reference. The results for UV-visible absorption spectroscopy showed characteristics of absorption peaks for non-metallic CNPs at 300 nm and Cu-CNPs at 550 nm. The CNPs nanoparticles showed agglomeration with rough porous surface morphology having sharp and pointed edges as revealed in FE-SEM analysis. Cu-CNPs showed spherical nano-disc morphology with irregular shape and surface topography. EDXS is an X-ray non-destructive analysis particularly used for the identification of the elemental profile of the tested material. The presence of metal ions (Cu) in the Cu-CNPs confirms it as a metal-conjugated nanoparticle. The hydrodynamic sizes of all the synthesized nanoparticles were measured through DLS analysis and it was observed that all the nanoparticles lie between 50 to 500 nm in range. The average size of non-metallic nano chitosan ranges between 40-70 nm. The hydrodynamic size of Cu-CNPs showed a size between 75-520 nm.

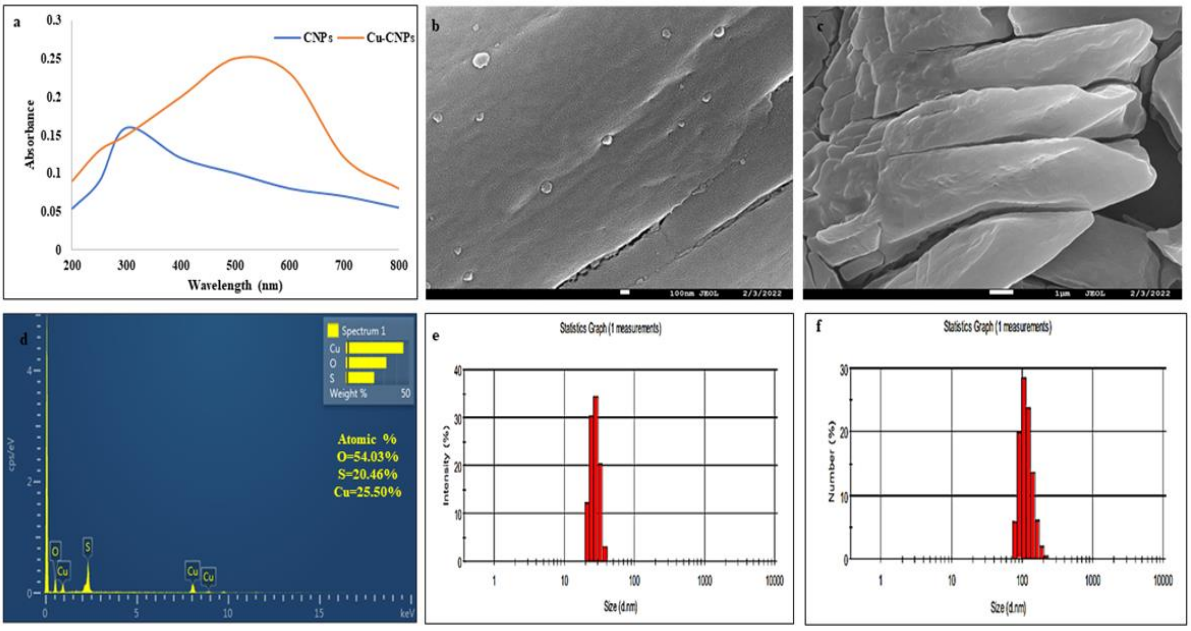

Fig. 1 Characterization of CNPs and Cu-CNPs through (a) UV-Vis spectra; FE-SEM analysis of (b) CNPs and (c) Cu-CNPs; (d) EDXS analysis of Cu-CNPs; DLS analysis of (e) CNPs and (f) Cu-CNPs

Poisoned Food Assay

The antifungal efficiency of both the nanoparticles were evaluated by poisoning the growing media of the fungal pathogen with chitosan-based nanoparticles. This assay reveals that in comparison to CNPs, Cu-CNPs showed complete inhibition (100% PIRG) of *R. stolonifer* at 2000 µg/mL. While, CNPs treated plate at 2000 µg/mL showed

only 10% of fungal growth inhibition. The density of the fungal mat was found to be progressively reduced in Cu-CNPs treated plates with an increasing range of concentration. It was also observed that the formation of sporangium or the sporulation process of the pathogen was triggered by the increasing concentration of both the chitosan-based nanoparticles. The fact is already established that metal nanoparticles when combined with chitosan, the bioactivity is increased manifolds (Reddy et al., 2008). There are reports that chitosan and its metal-based nanoparticles are highly efficient in controlling the ramification of phytopathogens (Yanat and Schroen, 2021). Researchers have successfully explained that CU-based nanoparticles significantly inhibited the growth of a range of fungal pathogens (Ingle and Rai, 2016).

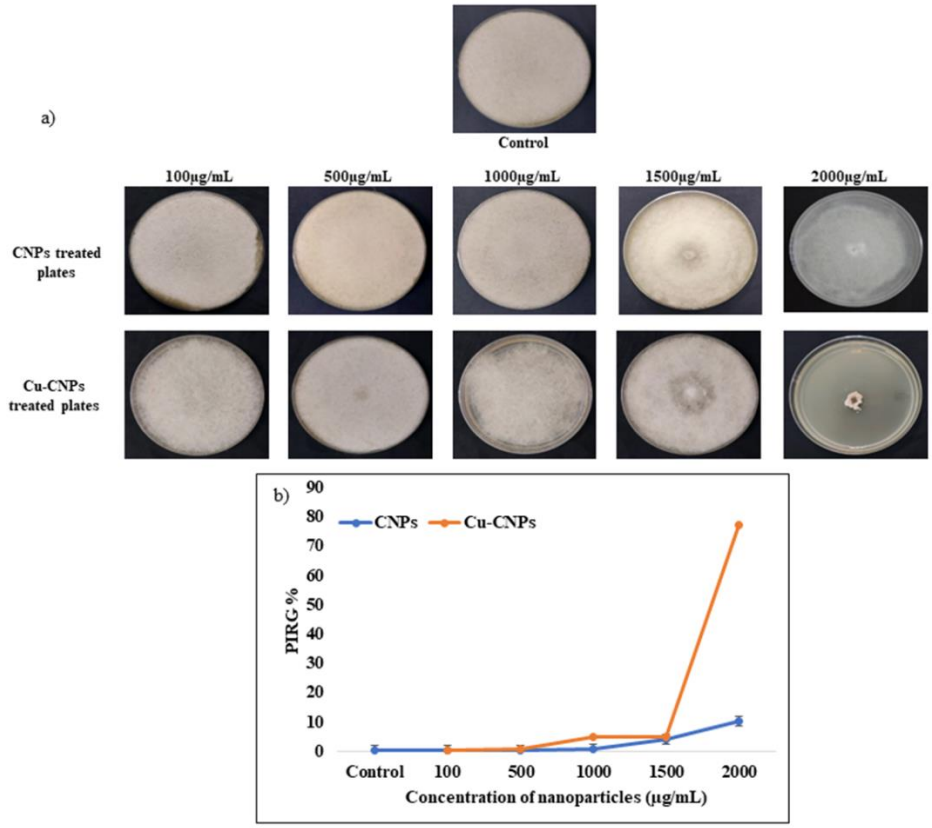

Fig. 2 Characterization of CNPs and Cu-CNPs through (a) UV-Vis spectra; FE-SEM analysis of (b) CNPs and (c) Cu-CNPs; (d) EDXS analysis of Cu-CNPs; DLS analysis of (e) CNPs and (f) Cu-CNPs

Spore viability assay

Spores of *R. stolonifer* treated with Cu-CNPs were found non-viable at 2000 µg/mL. The viability percentage of spores treated with 2000 µg/mL of Cu-CNPs was 1.88%. On the other hand, CNPs treated spores showed 12.56% of viable spores at the highest treated concentration. 50% of the spores were found viable at 1500 µg/mL of Cu-CNPs. Whereas, the untreated spores showed 100% viability in the growth medium. A number of scientists have proved that metal and metal-derived nanoparticles stand as strong antifungal agent (Chen *et al*., 2016). Research by Triawan et al., 2015 revealed that when spores of *R. stolonifer* are treated with magnesium oxide and zinc oxide nanoparticles, the viability of the spores and their propagation is completely inhibited. In order to completely triggered the propagation of a phytopathogen, it is important to trigger its propagative unit i.e., spore (Judelson and Blanco, 2005). The viability of the spore is sensitive to various abiotic stresses (Li et al., 2010). The application of the synthesized Cu-CNPs can significantly generate cellular stress and check the viability of the spore.

Lipid Peroxidation of R. stolonifer

The fungal tissue treated with 2000 µg/mL of Cu-CNPs showed 80% of MDA production as a result of lipid peroxidation due to nanoparticle treatment. Alike of Cu-CNPs, CNPs also showed 75% of MDA production in the fungal tissue. It was also observed that at each treated concentration of both the nanoparticles, the production of MDA was found higher in Cu-CNPs treated fungal tissue in comparison to CNPs treated fungal tissue. In

contrary, the untreated fungal tissue showed no MDA production. The fungal tissues are composed of polyunsaturated fatty acid which contains methylene groups in them. These methylene groups are highly affected by free radicals or ROS which are produced in the fungal tissue as a result of the application of nanoparticles. ROS generated in the fungal tissue results in the oxidation of fungal membrane lipids thereby causing membrane destabilization. The oxidation of the membrane lipid produces an aldehyde product called MDA. The more is this generation of stress in the fungal tissue upon exposure of the nanoparticles the more will be the MDA content in the fungal tissue (Kalagatur et al., 2018). Our result depicts that Cu-CNPs can significantly elevate the MDA content in the fungal tissue with increasing concentration.

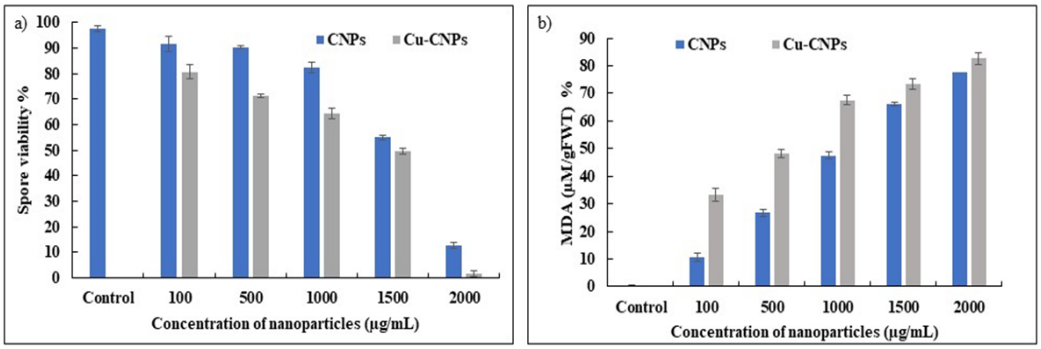

Fig. 3 Effect of different concentrations of CNPs and Cu-CNPs on (a) spore viability percentage and (b) MDA percentage of *R. stolonifer*

Analysis of oxidative stress production on R. stolonifer

The generation of oxidative stress in the fungal mycelium was determined through DCFH staining method. The fungal mycelium incubated with Cu-CNPs showed maximum intensity of fluorescence due to the generation of cellular oxidative stress. The fungal mycelium treated with CNPs showed moderate intensity of fluorescence as a result of the generation of minimal oxidative stress in the mycelium. The fungal mycelium without any treatment showed no fluorescence when observed under the microscope. This assay significantly proved that Cu-CNPs at 2000 µg/mL generate maximum cellular oxidative stress in the fungal pathogen in comparison to CNPs. The emission of high intensity of fluorescence is directly proportional to the generation of oxidative stress in the fungal pathogen (Kumar et al., 2016; LeBel et al., 1992). The level of fluorescence emitted in the fungal mycelium treated with Cu-CNPs hindered the integrity of the fungal membrane and thereby affect the functionality of the pathogen (Kalagatur et al., 2018).

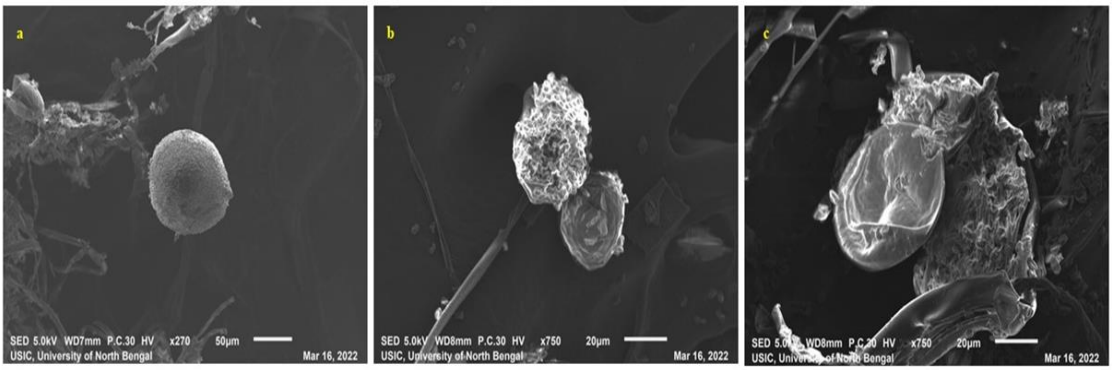

Figure 4. Morphological changes of *R. stolonifer* sporangium directly exposed to (a) distilled water; (b) CNPs (2000 µg/mL); (c) Cu-CNPs (2000 µg/mL) observed under scanning electron microscope (SEM)

Analysis of ultrastructural changes on sporangium of R. stolonifer

Under Scanning Electron Microscopy (SEM), it was observed that the application of Cu-CNPs at 2000 µg/mL produces define damages in the sporangium and spores of *R. stolonifer*. The sporangium membrane was completely damaged due to protoplasmic leakage and generation of membrane destabilization as a result of Cu-CNPs treatment. CNPs at 2000 µg/mL was observed to create moderate damage in the sporangium and its membrane. No membrane damages were observed in the sporangium of the untreated set. It contains completely intact undamaged sporangium.

Irreversible damages are produced in the sporangium of the pathogen as a result of the treatment of nanoparticles. Due to membrane destabilization, cellular protoplasmic leakage occurs that produces various surface abnormalities as a result of which the Cu-CNPs treated sporangium looks like a punctured ball (Dananjaya et al., 2017a). The polycationic molecule chitosan contains multiple positive charges in it. Due to the amalgamation of Cu ion into the chitosan network the resulting molecule is highly positively charged in nature. On the other hand, the fungal membrane contains negatively charged membrane components. The interaction between super positively charged nanoparticles and negatively charged fungal cellular components leads to ionic imbalance (Goy et al., 2009). It is suggested that chitosan nanoparticles enter into the fungal cell and binds with the fungal DNA, thus inhibiting protein synthesis (Kulikov et al., 2014).

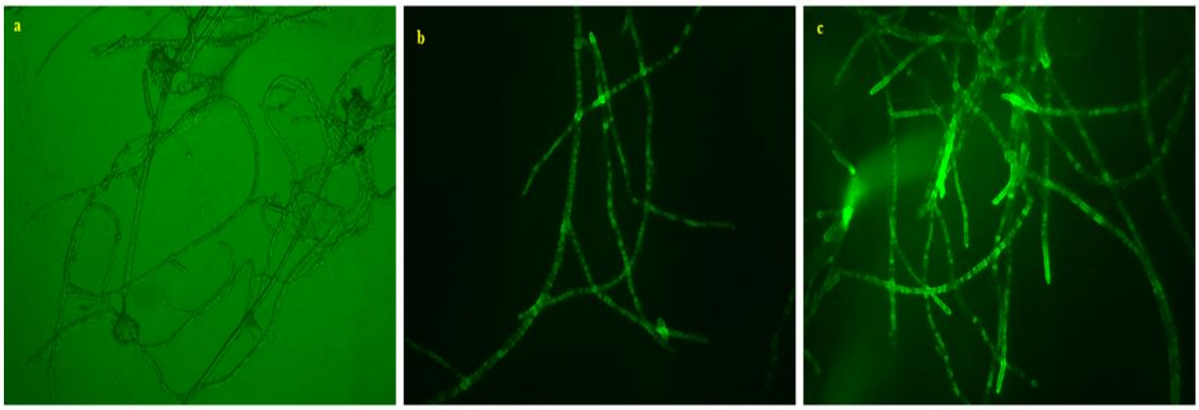

Figure 5. Fluorescence microscopic observations showing generation of oxidative stress in *R. stolonifer* mycelium due to the effect of (a) distilled water; (b) CNPs (2000 µg/mL) and; (c) Cu-CNPs (2000 µg/mL)

Conclusion

CNPs and Cu-CNPs were successfully synthesized by ionic gelation method and characterized as active nanoparticles. The application of Cu-CNPs against *R. stolonifer* showed progressive reduction of the growth of the pathogen. At 2000 µg/mL, Cu-CNPs completely terminates the growth of the pathogen in PDA media. At the same concentration the spores of the pathogen were found completely non-viable. Cu-CNPs generates maximum MDA level in the fungal tissue as a result of the peroxidation of the membrane lipids. The fluorescence assay further confirms the generation of oxidative stress through the emission of high intensity of fluorescence. Observations under SEM concludes that Cu-CNPs produces defined membrane damages of *R. stolonifer*. Thus, it can be concluded that Cu-CNPs can be used as a potential antifungal agent against the virulent phytopathogen, *R. stolonifer*.

Acknowledgement

The authors would like to thank University Grant Commission (UGC), Government of India for providing basic research support. The first author is grateful to UGC for providing financial support through NET-JRF fellowship (672/ CSIR NET JUNE 2019). The authors are also thankful to DST-FIST, National Seeds Corp. Ltd., SAIF-IIT Bombay, CIF-LPU (India), Department of Botany (University of North Bengal, India) and Dept. of Zoology (University of North Bengal, India) for their co-operation.

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ISSN No. 0974-6927

NBU JOURNAL OF PLANT SCIENCES
Volume 14, 2022

Contents

Articles

Editor: Ex-officio Head (Prof. Monoranjan Chowdhury), Department of Botany, University North Bengal
Published yearly by the Registrar, University of North Bengal
Printed at University Press