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RESEARCH ARTICLES

Bioscience Biotechnology Research Communications

Open Access International Journal

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Biosc Biotech Res Comm is an official publication of an academic, non-profit Society for Science and Nature, Bhopal India, since 2008. It is a peer reviewed journal that publishes original research articles pertaining to exciting applied areas of biology including biomedical sciences. The aim of *Biosc Biotech Res Comm* is to promote quality scientific research in fundamental and applied areas of biological and biomedical sciences and their allied branches.

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Editors Communique

Have we tamed the coronavirus? May be yes,
as pandemics do not die, they can only be faded !

Science and technology has made it possible, in the shortest span of time, it has shown that with firm determination and international cooperation, we can win over the onslaughts of even the worst of the pandemics. COVID-19 is perhaps fading over now, due to our coordinated efforts worldwide. Though we have lost millions, in the two year period, partly due to the mishandling of the viral attacks and somewhat by our own follies and carelessness. Anyway lessons learnt from the past, always make us more stronger and determined. Let us now not relax and work on a better mode, as all is still not well yet. The almost taming of the virus and its cousins have indicated some of the concealed failures, on which we have to focus now. We have to be more vigilant, and even a bit of laxity can spoil the good work done. On societal and governmental parts, utmost care and caution is required on a long term basis.

On behalf of Bioscience Biotechnology Research Communications, we falter at words to express our deep sense of solitude and grief on the catastrophic events of the world wide pandemic, spanning over two years now. We pray for the strength to bear this universal calamity and come up with long lasting fortitude to eradicate it soon.

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Sharique A. Ali, PhD
Editor-in-Chief

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Optimizing Genome Editing for Wheat Genetic Improvement: Current Status and Future Prospective

Fekede Meshesha Namo
Department of Biotechnology,College of Agriculture and Natural Resource,
Debre Berhan University, P. O. BOX 445 Debre Berhan, Ethiopia

ABSTRACT

Wheat (*Triticum aestivum L*) is the main source of nutrition and feeds more than 30% of the world's population. It is grown in various environments, providing most humans with around 20% of their calories and protein. Genome editing holds countless promise to accelerate the development of improved crop varieties including wheat via providing powerful tools to modify the genomic regions that controlling major agronomic traits. CRISPR/Cas9 system is an effective method for targeted genome editing. CRISPR/Cas9 allows researchers to target multiple homoeoalleles simultaneously and it enables the production of targeted mutations in all copies of a gene. The CRISPR-Cas and associated technologies derived from the naturally occurring prokaryotic CRISPR immune system and it has been used to target multiple homoeoalleles simultaneously and accelerate progress in functional genomics and molecular breeding in wheat. CRISPR/Cas9 is widely used to improve agricultural traits by knocking out unwanted genes or genes conferring undesirable phenotypes.

Targeted knockout wheat with foreign DNA is generated in the T0 generation. Ultimately, the foreign DNA can be segregated by selfing and crossing. A targeted gene-modified plantlet without foreign DNA is generated in the T0 generation. This approach has been reported in wheat for the first time. DNA free genome-edited wheat plants have been generated. Genes required for genic male sterility are identified, CRISPR/Cas9- mediated disruption of these genes will enable the rapid production of male-sterile wheat. This represents a promising method for manipulating recessive sterility genes to capture heterosis in wheat. CRISPR-based genome editing will bring functional genomics and rational design-based molecular breeding of polyploid wheat to the forefront of wheat biology. Transgene-free, gene edited wheat will play a critical role in addressing environmental issues while promoting sustainable agriculture.

KEY WORDS: CRISPR/CAS9, DNA, EDITING, GENOME, IMPROVEMENT, WHEAT

INTRODUCTION

Wheat (*Triticum aestivum L*) is the main source of nutrition and feeds more than 30% of the world's population (Wang et al., 2019). The demand for wheat with high-quality traits has increased globally due to the growing population and the rising living standards in countries worldwide (Kumar et al., 2019). The presence of wheat gluten gives the dough viscoelasticity and ductility, and it can be processed into a variety of foods to meet people's needs (Veraverbeke and Delcour, 2002). Common wheat is a keystone crop species. It is grown in various environments, providing most humans with around 20% of their calories and protein (Uauy et al. 2017); thus, it occupies an important position in food security. As the global population increases, improving the yield of wheat is critical to ensure future availability. Geneticists have exploited natural or artificial wheat variations for breeding. Indeed, conventional breeding approaches have played a major role in increasing grain yields and quality based on broad genetic variations in wheat (Nadolska-Orczyk et al. 2017).

However, wheat is an allohexaploid (2n = 6 9 = 42, AABBDD); it harbors three closely related sub genomes inherited from three homoeologous ancestors (Petersen et al. 2006). Thus, most wheat genes have three similar but not identical copies, with functional redundancy and complementarity among the A, B, and D genomes. As a result, the probability of the simultaneous mutation of genes in the A, B, and D genomes by natural processes or induced mutagenesis is very low. Therefore, the complex

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polyploid nature of wheat has hindered the development of functional genomics and breeding, especially compared to other cereals, such as rice and maize.

Genome editing holds countless promise to accelerate the development of improved crop varieties via providing powerful tools to modify the genomic regions controlling major agronomic traits. The actual deployment of these technologies for crop improvement requires further optimization of the components of the genome editing systems and tools for different applications. The functionality of the various components of the genome editing tools like CRISPR/Cas9 system was authenticated using the wheat protoplast transformation assay followed by the next-generation sequencing (NGS) of the targeted genomic regions. The wheat codon optimized Cas9 was revealed to be effective tool for the targeted gene editing in the wheat genome.

The recent emergence of genome-editing technologies has transformed basic biological studies and practical biotechnological applications. Among the major genome-editing tools such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated nuclease (Cas), CRISPRCas- mediated genome-editing technique has become the method of choice for most of the laboratories due to its simplicity, affordability, and high success rate. Although many laboratories around the world have rapidly adopted conventional CRISPRCas9-mediated approaches for targeted mutation and gene knockout via the non-homologous end-joining (NHEJ) repair, precise genome editing with CRISPR-Cas9 remains challenging to achieve (Molla and Yang, 2019).

Typically, precise changes in the genome require the use of homology-directed repair (HDR), which involves the supply of an exogenous donor template with the desired changes. However, the efficiency of HDR-mediated genome editing is extremely low in higher plants due to the difficulties in delivering adequate repair templates and the low rate of homologous recombination. Moreover, HDR occurs only during S and G2 phase of the cell cycle.CRISPR/Cas9 system is an effective method for targeted genome editing, and its efficiency has been shown in several plant species (Endo et al., 2015). Although this system is relatively easy to use and more precise compared to other genome editing technologies, there are still some issues, particularly for polyploidy plants, such as editing efficiency and off-target mutation rate. Here, a series of experiments were conducted to show the efficient genome editing with CRISPR/Cas9 system in wheat protoplast and the results confirmed that CRISPR/Cas9 system is a promising tool for further targeted editing of wheat genome (Jun et al., 2021).

The CRISPR-Cas9-mediated genome-editing technology provides unprecedented tools to precisely edit DNA sequences in animals and plants (Gao, 2018, Mali et al., 2013). This technology requires expression of the Cas9 protein, production of a guide RNA (gRNA) that complements the target DNA sequences, and the existence of an NGG protospacer adjacent motif (PAM) site in the target sequence (Cong et al., 2013, Mali et al., 2013). Briefly, genome editing mediated by CRISPR-Cas9 utilizes a 20-bp gRNA that directs the Cas9 nuclease to the target site by base pairing. Cas9 cuts the target site to generate a double-strand break (DSB). Mutations are introduced during the DNA repairing process. Because of its simplicity, CRISPR-Cas9 has been widely adopted. Several CRISPR vectors have been developed for genome editing in plants (Ding et al., 2018). It was proven that CRISPR-Cas9-mediated genome editing technology could successfully generate various heritable mutations in plant species (Bortesi et al., 2016).

After transformation, the first mission is to isolate primary transformants with expected mutations. To this end, restriction enzyme digestion or sequencing of the PCR amplicons is typically performed (Bortesi et al., 2016). However, both methods are time- and money consuming and laborious. Next, mutants without the T-DNA insertion may need to be identified, which are favored in both basic and applied researches because of the following reasons. First, prolonged existence of CRISPR-Cas9 in the mutants would greatly increase the risk of producing off-target mutations. Second, transgene-free materials are more easily accepted by the public. Cas9-free mutants could be obtained by self-crossing or backcrossing. In B. napus, for instance, by using gene-specific primers (such as Cas9), 10.9% (58/530) of the mutant plants in the T1 generation lost the Cas9 transgene by self-crossing (Yang et al., 2017). a ratio lower than what is expected, which need larger populations to get Cas9-free mutant. Therefore, an easy method to screen for the mutants in need in both primary transformants and their offspring is highly required.

Application of the CRISPR/Cas9 system requires the DNA sequences of the target genes. Given the availability of the annotated wheat genome and the elucidation of a growing number of genes controlling important agronomic traits in other plants, it is easy to isolate orthologous genes in wheat based on homology based cloning. In addition, CRISPR/Cas9 allows researchers to target multiple homoeoalleles simultaneously and it enables the production of targeted mutations in all copies of a gene; thus, the system holds great promise in the characterization of genes endowing important agronomic traits in polyploid wheat. Furthermore, it has been used to modify multiple genes controlling different agronomic traits in wheat. This technology will bring a new dawn to wheat biology and breeding programs. In this review, we briefly outline the utilization of the CRISPR/Cas9 system, with an emphasis on the most important breakthroughs thus far.

CRISPR/Cas9 contains two major components: a sgRNA, which is responsible for recognizing target DNA, and the Cas9 endonuclease, which is responsible for generating DSB at predesigned target DNA site. Cas9 from Streptococcus pyogenes (SpCas9) was the first well-characterized RNA-guided endonuclease. It is a multifunctional protein that contains two nuclease domains: the HNH domain and RuvC-like domain. Each of them cuts one DNA strand, generating blunt-end DSBs; this triggers endogenous DNA repair systems, resulting in targeted

mutants. The only prerequisite for applying CRISPR/Cas9 to a given site is the presence of a protospacer-adjacent motif (PAM; NGG for SpCas9) next to the sequence of interest. For different target sites, Cas9 is constant; we can only change the guide sequence in the sgRNA.

Crop improvement aims to increase crop yield and resistance to biotic and abiotic stress, as well as quality and nutritional value. Crop yield has been significantly increased through advanced agricultural technologies over several decades. Crop quality has been a greater concern of consumers since it is directly associated with human health by providing multiple nutrients such as proteins, fiber, vitamins, Genome editing can create predictable and inheritable mutations in specific sites of genome, with the lowest probability of off-target and no integration of exogenous gene sequences. GE-mediated DNA modifications encompass deletions, insertions, single nucleotide substitution (SNPs), and large fragment substitution. Four site-directed nuclea (SDN) families are involved in a nucleotide excision mechanism: homing endonucleases 0or mega-nucleases (HEs) (Cohen et al., 1998), Zinc-Finger Nucleases (ZFNs) [Bibikova, et al., 2002), transcription activator like effector nucleases (TALENs) (Christian et al., 2010) and CRISPR-associated protein (CAS) (Cong et al., 2013).

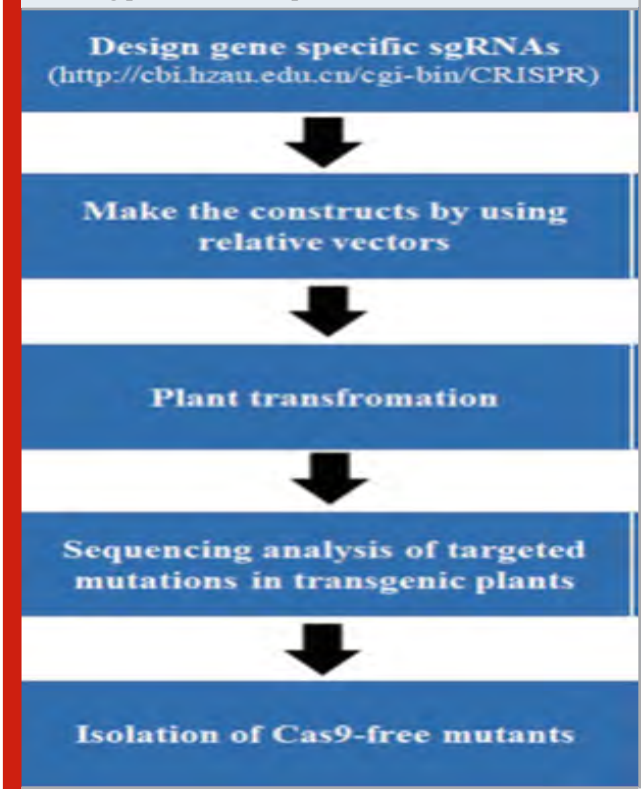
The number of cases in crop improvement using GE has increased significantly. Among the various target traits for crop improvement, crop quality is one of the highest objectives. Here, we summarized the recent progress in CRISPR/Cas9-mediated crop quality improvement and provide further discussion on the future application of GE

CRISPR/Cas9 Gene-Editing System in Plants: CRISPR/Cas systems have been divided into two classes and five types according to the classification of the CAS protein. The type II CRISPR/SpCas9 system from Streptococcus pyogenes has been modified and developed as versatile GE tools for different applications (Hsu et al., 2014). It consists of two core components: the guide RNA (gRNA or sgRNA) and the Cas9 protein. The gRNA constitutes CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The former contains a~20 nt fragment (also known as a spacer, complementary to a specific site of target genes), followed by a protospacer adjacent motif (PAM) in the target genes of interest. Under the guidance of gRNA, Cas9 nuclease creates DSBs at ~3 bp upstream of the PAM motif (Jinek, et al., 2012).

The cleavage repaired in NHEJ way, usually results in gene knockout or loss of protein function (Liu et al., 2019). Alternatively, when an exogenous DNA repair template is provided, HDR can be triggered, resulting in the introduction of the repair template into a target genomic region [9]. In plants, CRISPR/Cas9-based gene-editing consists of multiple steps including the selection of target sites, designing and synthesis of sgRNA, delivery of transformation carrier or ribonucleoprotein (RNP) in plant cells, transformation, and screening of gene-edited plants. At present, the plant CRISPR/Cas9 and its derived system have shown various genome-editing ability, such as gene knock-in, knockout, knockdown, and expression activation as well. In addition, simultaneous editing on multiple genes has contributed to pathway-level research.

The CRISPR-Cas and associated technologies derived from the naturally occurring prokaryotic CRISPR immune system are definitely revolutionary in studying basic biology and manipulating genomes of diverse organisms. These robust, reproducible, and easy-to-use technologies allow the manipulation or modification of genome in several ways, including, but not limited to (a) by simply incorporating random mutation (insertion or deletion) through non-homologous end joining to disrupt gene(s); (b) by generating targeted point mutations in genes using precise base editors; and (c) by a whole gene insertion employing the cell's homology directed repair pathway. Improvement in protocols, higher access to CRISPR-Cas tools, and necessary changes in the global regulatory environments are needed for the broader application of this frontier technology in diverse areas like production of energy, health, environment, medicine, and sustainable food production in changing climate (Tofazzal et al., 2020).

Figure 1: The workflow used in this study is workflow of generation CRISPR-Cas9 genome-editing transgenic plants, from target sgRNAs designing, plant transformation, and isolating positive mutant plants



Analyzing genome editing events by next-generation sequencing (NGS): The transient expression in the transformed protoplasts provides a simple and rapid method for assessing the editing capability of the CAS9/gRNA constructs (Upadhyay et al., 2013, Shan et al., 2013). This approach has adopted to screen gRNAs for

the ability to generate Cas9-mediated changes in the wheat genome. Further, the protoplast expression assay was combined with NGS for the rapid and cost-effective analysis of multiple genomic regions. This strategy was used to evaluate the gRNAs designed to target four genes controlling domestication (Q gene) (Simons et al., 2006), seed development (*TaGW2*) (Su et al., 2011) and disease resistance (*TaLpx-1* and *TaMLO*) phenotypes in wheat (Wang et al., 2014, Nalam et al., 2015).

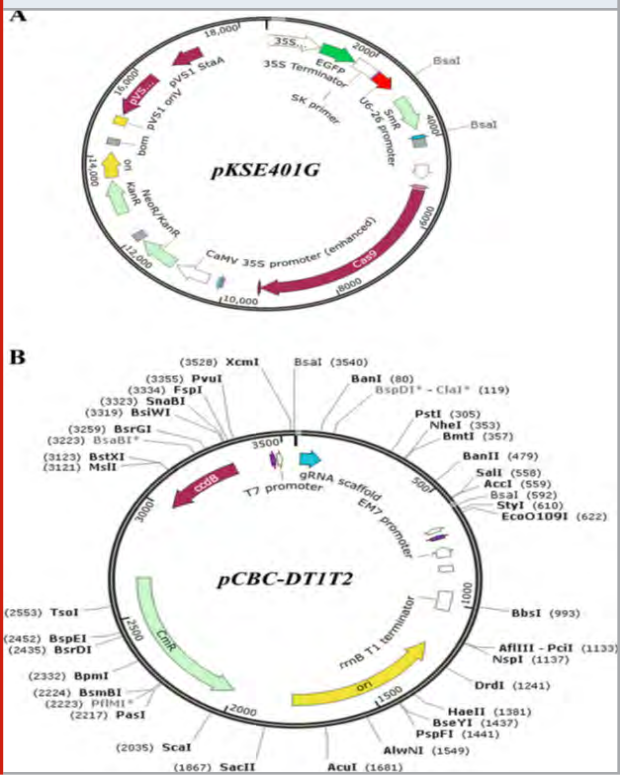
The editing specificity of the designed gRNAs was certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission. The A and D genome copies of the *Q* gene were successfully targeted by three gRNAs on the 5'-end, and only the A genome copy was targeted by two gRNAs on the 3'-end. Only one of the two target sites in *TaGW2* was edited in all three wheat genomes. Both gRNAs designed for the *TaLpx-1* gene produced deletions in the B and D genomes, whereas no editing events were detected for the target in the A genome due to its high divergence from the guide sequence. The gRNA targeting *TaMLO* induced mutations only in the wheat a genome, as previously reported (Wang et al., 2014).

Figure 2: Design of the primer sets containing gene-specific sgRNAs. (a) At the "submit" page, select a genome you are studying, and input gene locus, chromosome position, or DNA sequence in FASTA format. Other parameters are set as default. A few seconds after submission, the result is shown in web browser, which contains sgRNA sequence information, including GC content, on-target score, off-target score, and restriction endonuclease sites. (b) Choose the sgRNA sequences, and replace 19-nt N in the forward primers with 19-nt target sequences in front of PAM (NGG) and 19-nt N in the reverse primers with reverse complement sequences of 19-nt target sequences in front of PAM (NGG)

Current Status of Genome Editing in Wheat: CRISPR/Cas9 has been used to target multiple homoeoalleles simultaneously and it will accelerate progress in functional genomics and molecular breeding in wheat.

Generating CRISPR-edited DNA-free wheat: CRISPR/Cas9 is widely used to improve agricultural traits by knocking out unwanted genes or genes conferring undesirable phenotypes. However, this process usually involves transgenic intermediates, which causes regulatory concerns and is not accepted worldwide (Zhang et al. 2020). For public acceptance, gene removal or bypassing foreign elements to edit endogenous genes is a good choice (He and Zhao, 2020). Based on the reagents needed for CRISPR-mediated editing, there are two main ways to produce CRISPR-edited DNA-free plants.

Figure 3: Construction of pKSE401G. (a) The CRISPR-Cas9 vector pKSE401G is modified from pKSE401 (Xing et al., 2014). The 35S-sGFP-terminator cassette is amplified from pK7GWIWG2D 30.(Karimi et al., 2007) using primers 35S-GFP-Ter-F and 35SGFP- Ter-R and inserted into the PmeI site of pKSE401 by the Gibson Assembly method (Gibson et al., 2009). (b) The map of pCBC-DT1T2 (Xing et al., 2014)

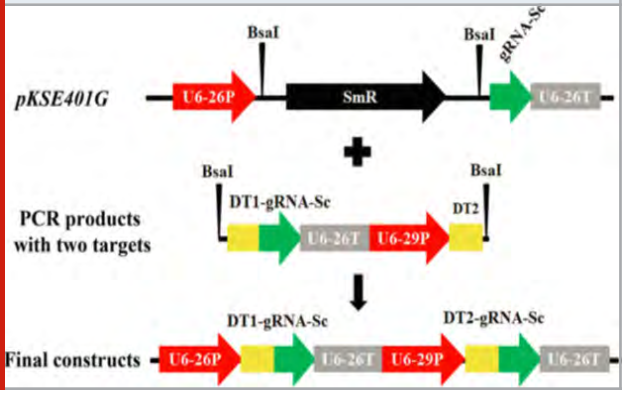


In the vector-based method, a vector is delivered into wheat callus using Agrobacterium or particle bombardment. It then integrates into the genome and the encoded genome editing elements are expressed, enabling targeted gene knockout. Targeted knockout wheat with foreign DNA is generated in the T0 generation. Ultimately, the foreign DNA can be segregated by selfing and crossing. For example, researchers created a triple-knockout mutant of TaQsd1 via Agrobacterium delivered CRISPR/Cas9. The mutant was then crossed with wild-type wheat plants, producing transgene-free triple-recessive TaQsd1 mutants that exhibited longer seed dormancy (Abe et al., 2019). Similarly, a marker free wheat mutant was obtained among the offspring of T0 plants (Wang et al., 2017).

Sometimes, vectors are not integrated into the genome; instead, they may transiently express their encoded genome editing elements to knock out genes. A targeted gene-modified plantlet without foreign DNA is generated in the T0 generation. This approach has been reported in wheat for the first time. Researchers delivered vectors containing CRISPR/Cas9 elements into wheat callus through particle bombardment; the plantlet was subsequently regenerated without antibiotic selection. This transient expression-based CRISPR/Cas9 system produced transgene-free, homozygous mutants (Zhang et al., 2016). In addition, transgene-free wheat carrying nucleotide substitutions have been generated by transiently expressing CBEs or ABEs (Zong et al., 2017; Li et al., 2018).

DNA free genome-edited wheat plants have been generated. Though the editing efficiency was lower, the specificity was higher than with a vector-based system (Zhang et al., 2016). Moreover, nCas9-PBE mRNA and sgRNA were transcribed in vitro and delivered into immature wheat embryos. DNA-free base editing at TaALS-P174 was obtained, endowing wheat with resistance to the herbicide nicosulfuron (Zhang et al., 2019). In addition, Cas9 can be expressed in vitro and assembled with the sgRNA into a Cas9/sgRNA ribonucleoprotein, which is delivered into immature wheat embryos by particle bombardment. The ribonucleoprotein cleaves the target site immediately and is quickly degraded, generating DNA-free edited wheat (Liang et al., 2017).

Figure 4: The gRNA modules used for the assembly of two gRNA expression cassettes. Examples of the assembly of two-gRNA expression cassettes for dicots using the gRNA modules. Using pCBC-DT1T2 as the template, two AtU6 promoter-sgRNA-AtU6 terminator cassettes were amplified by PCR, and the PCR fragments were then inserted into pKSE401G by Golden Gate Assembly. U6-29p and U6-26p are two Arabidopsis U6 gene promoters; U6-29t and U6-26t, corresponding Arabidopsis U6 gene terminators with downstream sequences, respectively. gRNA-Sc, gRNA scaffold; DT1/2, dicot target-1/2



The final CRISPR-edited DNA-free products are similar to natural and artificial mutants, which are not subject to GMO regulations. We believe that this is the direction of future breeding , and we will play a vitel role in realizing sustainable agriculture in future.

Future Perspectives: The development of CRISPR/Cas9 technology has been extensively used in wheat genome editing. This technology permit multiplex genome engineering, which has enabled the production of loss-of function triple wheat mutants; thus, it is a powerful tool for introducing desired traits conferred by a loss-of function mutation into commercial cultivars through NHEJ. As additional genes required for genic male sterility are identified, CRISPR/Cas9- mediated disruption of these genes will enable the rapid production of male-sterile wheat. This represents a promising method for manipulating recessive sterility genes to capture heterosis in wheat.

CRISPR-mediated precise genome editing is a useful means to achieve these targeted substitutions and replacements by modifying endogenous genes without introducing linkage drag; it can also introduce new alleles (segregating as a single locus) into a predetermined genomic site. Thus, this approach could accelerate the breeding process. Nowadays, CRISPR-mediated precise genome editing is a useful means to achieve these targeted substitutions and replacements by modifying endogenous genes without introducing linkage drag; it can also introduce new alleles (segregating as a single locus) into a predetermined genomic site. Thus, this approach could accelerate the breeding process. Trans-generational CRISPR/Cas9 activity has been used to modify multiple target sites in tomato and wheat (Rodriguez-Leal et al. 2017; Wang et al. 2018). This recommends that valuable, desired phenotypes in elite wheat germplasm, which are recalcitrant to transformation, could be induced by crossing with lines carrying CRISPR/Cas9 elements. In addition, wheat genes have been successfully edited through pollination using CRISPR/Cas9-transgenic maize as a haploid inducer (Budhagatapalli et al., 2020).

Such haploid induction-mediated genome editing would not only reduce the genotype dependence on site-specific mutagenesis in wheat, but also provide a path to produce transgene-free gene-edited inbred wheat lines. Collectively, these technologies will accelerate wheat breeding. Some studies have reported that although CRISPR/Cas9 can cleave a target site, sometimes it also cleaves sites with a few mismatches to the target site. This off target effect is a major concern in gene therapy, but this issue might not be a barrier in plant biotechnology. The putative off-target mutation could be eliminated through back-crossing or crossing with wild-type plants. Moreover, it is advisable to design target sites using web-based tools to reduce off-target mutations by leveraging computation.

Combined with other achievements, including the production of high quality genome sequences and improved transgenic methods, CRISPR and CRISPR-based genome editing will bring functional genomics and rational design-based molecular breeding of polyploid wheat to the forefront of wheat biology. We believe that transgene-free, gene edited wheat will play a critical role in addressing environmental issues while promoting sustainable agriculture. Significantly, it is not a replacement for traditional breeding; it is just one of the methods advancing wheat breeding programs and accelerating wheat biology.

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Biotechnological Communication

Effect of Calcium Carbide Exposure Through Inhalation in Lungs of *Mus musculus*

Soumi Banerjee[1#], Pujita Ghosh[1#], Debajyoti Patra[2], Pratip Chakraborty[3], Kaustav Dutta Chowdhury[1*]

[1]Cell and Molecular Biology Laboratory, Department of Zoology, Rammohan College, Raja Rammohan Sarani, Kolkata, India.
Molecular Biology and Tissue Culture Laboratory, Post Graduate
[2]Department of Vidyasagar College, Vidyasagar College, Kolkata, India.
[3]Department of Infertility, Institute of Reproductive Medicine, Kolkata, India.

ABSTRACT

Study on occupational injuries indicates the industrial exposure to air-pollutants, asthmagens, carcinogens, and noise for extended hours as leading risk factors directing to death. This exposure generally occurs by inhalation, ingestion, or via dermal contact. Out of which inhalation is the most rapid route of uptake through breathing in the air that is contaminated with particulate matter/dust, vapours of volatile or semi-volatile contaminants and aerosols due to outdoor and indoor industrial activities. Irritational lung injury, asphyxia, respiratory depression, tachycardia, pulmonary edema may develop as long-lasting systemic effects even after completion of the working life of a worker.Most occupational lung diseases are caused by repeated, long-term exposure. Therefore, our study was conducted to analyze the effect of 40 days of chronic calcium carbide exposure in a close chamber through inhalation in lung of Swiss-albino mice. ALT, AST, SOD and catalase activities were estimated spectrophotometrically. Spectrofluorimetric estimation was performed for reactive oxygen species determination. Flow cytometric analysis was performed to examine cell death and cell cycle. Pro-apoptotic and anti-apoptotic protein levels were estimated by immunoblot. Data demonstrated altered body homeostasis as marked by AST/ALT assay. 3gm CaC_2 exposure indicated activation of antioxidant enzymes, increased cell death causing sustained animal survivability. 5gm and significantly 7gm CaC_2 exposure displayed antioxidant enzymatic activities along-with decreased cell death and animal survivability. While in 9gm CaC_2 exposure total antioxidant enzymes were collapsed with increased cell death leading to probably maintenance of animal survivability to some-extent in the said group.

KEY WORDS: CAC2, CELL DEATH, LUNGS, MICE, ROS.

INTRODUCTION

Recent time witnessed an increase in respiratory distress due to environmental pollution, lifestyle as well as occupational exposures. In this context, the lung is the most affected organ due to its delicate endothelial network being constantly involved in gaseous exchange with the environment. Report suggests that 1 in 20 people suffers from chronic respiratory diseases (CRDs) globally, attributing CRDs as the third leading cause of death in the world (Momtazmanesh et al., 2019).

Amongst all other causes of CRDs, professional hazard (i.e.,breathing in chemicals, dust or noxious gases in industrial zones), is the most overlooked and neglected one. Occupational lung diseases may take a long time to develop and may have lasting effects on lungs even after the worker stops working. According to the World Health Organization (WHO), 125 million people worldwide are exposed to asbestos at work. According to global estimates, at least 90,000 people die each year from asbestosis, asbestos-related lung cancer and mesothelioma (Chen et al., 2022). Despite all efforts to prevent silicosis, it still afflicts tens of millions of workers and kills thousands of people every year, all over the world (Hoy et al., 2022).

Calcium carbide (CaC_2) also known as calcium acetylide being a source of acetylene and other noxious gases is considered as hazardous by the OSHA Hazard Communication Standard (29 CFR 1910.1200). It is mainly used to manufacture acetylene and other industrial compounds. Pure CaC_2

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produces acetylene when it reacts with water. Commercial grade CaC_2 is used in welding, desulphurization of steel, production of cyanamide and this grade of CaC_2 contains impurities like arsenic, sulfur and phosphorus which emits harmful gaseous compounds when dissolved in water (Bini et al., 2021, Okeke et al., 2022).

CaC_2 generated acetylene which is an analogue of ethylene is vastly used as fruit ripening agent in many developing countries as it is cheap and easily available (Maduwanthi and Marapana, 2019, Okeke et al., 2022). Reports suggest that ingestion of CaC_2 through edibles may affect the neurological system via inducing prolonged hypoxia (Okeke et al., 2022). Moreover, calcium carbide being an alkali compound causes irritation in mouth, nasal pathways, gastric discomfort, as well as damages mucosal tissues in abdomen (Okeke et al., 2022).

Figure 1: Change in survivability, body weight, organ weight and cell count along the course of calcium carbide (CaC_2) exposure.

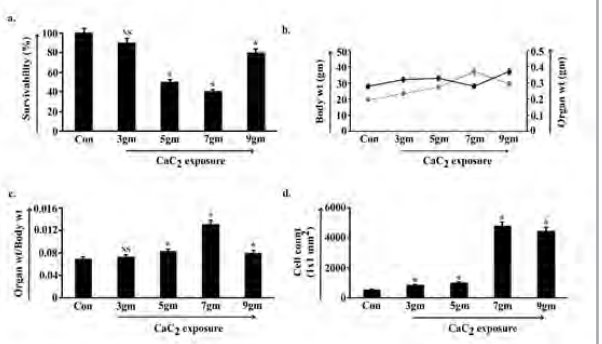
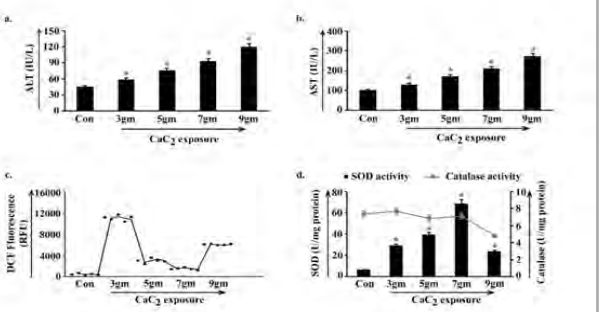


Figure 2: Estimation of oxidant-antioxidant homeostasis in the body along the course of calcium carbide (CaC_2) exposure.



Unwitty handling of CaC_2 may also cause ocular burn injury to blindness (Bandyopadhyay et al., 2013). The range of hazards also includes dizziness, fatigue, difficulties in breathing, seizures. It has been reported that exposure for three weeks with crude acetylene has possible deleterious effects on heart (Grek et al., 1976), liver and kidney (Frang et al., 1967) as well as blood constituents. Survey from a welding industry also reported that the exposure of acetylene might have been increasing the risk of respiratory cancer among the workers (Riboli et al., 1983).Even though CaC_2 shows potent toxicity through contaminated ingestion, much significant data is unavailable about its effect from direct inhalation which is common in industrial zones.

Considering the industrial atmosphere which is usually full of various fumes and other gaseous substances, occupational exposure to some extent is unavoidable for workers especially through respiration, in spite of proposed preventive measures. Regarding that, our study has been conducted to mimic such ambience and to investigate how chronic exposure of CaC_2 and CaC_2 generated obnoxious gases brings out deleterious effects in lungs.

MATERIAL AND METHODS

The technical grade calcium carbide (CaC_2) was obtained from Sigma-Aldrich (21039). Rest of the reagents were obtained from Sigma-Aldrich.

Animal maintenance & Schedule for exposure: Animal experiments were carried out in male Swiss albino mice (*Mus musculus* of 4-6 weeks age; 20-25g) maintained at 27 ± 2 °C with relative humidity of 44–56% and in 12 h light/darkness cycle as well as free access of food and water in a cross-ventilated room. All animal experiments were performed following the ethical guidelines of the Institutional Animal Ethics Committee (IAEC), Rammohan College, Committee for Control and Supervision of Experiments on Animals (CCSEA), MoFAHD DAHD, Government of India. Experiments were designed to minimize animal suffering and to use the minimum number necessary for valid statistical evaluation.

All animals were separated into two groups, one CaC_2 exposed group and a control group. The exposed group was further divide into four groups which were exposed to 3gm, 5gm, 7gm and 9gm of calcium carbide per day with requisite volume of water (w:v :: 1:10) for 15 minutes in a leak proof glass container of 24 litre volume. This exposure procedure was scheduled for 40days.

Survivability & Body weight-Organ weight Analysis: Animal survival was monitored daily and reported as the survivability (%) until 40 days. Each group consisted of five Swiss albino male mice (*Mus musculus*). Body weight and organ weight of every animal were recorded before sacrifice and after sacrifice respectively.

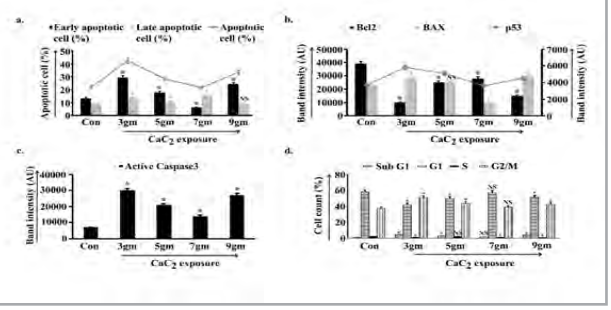
ALT and AST Activity: Blood was collected from animals and serum was prepared to estimate the activities of Aspartate transaminase (AST), alanine transaminase (ALT). Assays were performed following respective manufacturing kit protocols at room temperature. ALT and AST (TECO Diagnostics, CA, USA) activities were measured by estimating NADH oxidation at 320 nm wavelength for 30s intervals up to 2min.

SOD & Catalase: Superoxide dismutase (SOD) activity was measured from chloroform methanol extract following the standard protocol (Sengupta et al., 2014). Values were quantified spectrophotometrically (UV-1240 Pharma Spec,

Shimadzu, Kyoto, Japan) by calculating the changes in pyrogallol auto-oxidation at 420 nm in presence of catalase enzyme. One unit of SOD activity is equal to the 50% suppression of superoxide mediated oxidation of pyrogallol. Results were represented in unit/mg protein.Catalase activity was evaluated spectrophotometrically (UV-1240 Pharma Spec, Shimadzu, Kyoto, Japan) by measuring degradation of H_2O_2 in presence of tissue lysate as a source of enzyme. Values were quantified by measuring absorbances at 240 nm in 10s intervals. Data were represented in unit/mg protein (Sengupta et al., 2017).

Estimation of ROS: Cells of the lung were isolated from experimental groups by Collagenase-IV digestion. Intracellular ROS was measured by incubating 5% cell suspension with 5mM 2,7-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich, St. Louis, Missouri,USA), a fluorogenic dye, at 37°C for 15 min. After diffusion it was deacetylated by cellular esterase and turned into highly fluorescent 2',7'-dichlorofluorescin (DCF) upon oxidation. Emitted fluorescence (Ex:485 nm/ Em: 535 nm) was estimated in RF-6000 Fluorescence Spectro-fluorometer (Shimadzu, Kyoto, Japan). Values were presented by Relative Fluorescence Unit (RFU) (Sengupta et al., 2014).

Figure 3: Apoptotic parametric analysis along the course of calcium carbide (CaC_2) exposure



Flow Cytometric Analysis: Flow cytometric analysis was performed to analyze cell death and cell cycle by using FACS Calibur (BD Biosciences, Mountain View, CA, USA). Cells were suspended in Binding buffer (pH 7.4 containing 10mM HEPES–Na, 136mM NaCl, 2.7mM KCl, 2mM $MgCl_2$, 1mM NaH_2PO_4, 5mM glucose, 5mg/ml BSA and 2.5 mM $CaCl_2$). Annexin V-FITC and PI were then added to a concentration of 1µg/ml to each of the samples. Cell suspensions were then incubated for 15 min at room temperature in the dark. After incubation, samples were taken out for flow-cytometric analysis. Percentage of apoptotic cells were calculated using CellQuest software attached with the flow cytometer. (Sengupta et al., 2014).

In the process of cell cycle analysis, the samples were centrifuged at 500xg for 5 minutes. Supernatant was aspirated without disturbing the pellet. The cells were then washed by 1x PBS. Pellets were resuspended in 200µL 1x propidium iodide and RNase staining solution. The incubation was continued for 20-30 minutes in the dark at 37°C. In the next step, samples were placed in ice (still in dark) and cell cycle phases were determined by calculating PI fluorescence using flow cytometer.

Western Blot Analysis: Whole cell lysates were used as protein source for quantitative analysis of cellular Bcl2, Bax, p53 expressions and caspase3 activity. SDS-PAGE resolved proteins were transferred to the nitrocellulose membrane. Then the membrane was blocked in 3% BSA in TBST (50 mmol/L Tris-HCl, pH 7.5, 150mmol/L NaCl, 0.1% Tween20) and subsequently incubated with respective primary antibodies (Santa Cruz Biotechnology) (1:500 to 1:1000 dilutions in TBST). Next the membrane was incubated with alkaline phosphatase tagged secondary antibody (Santa Cruz Biotechnology) (1:2000 dilutions in TBST) and binding signals were visualized with NBT-BCIP. Respective band densitometric analysis was performed with ImageJ software (NIH, Bethesda, MD, USA).

Cell Count: Cell count was performed by calculating cell number using ImageJ software in the 1mm x 1mm area of the histological section of lung tissue. The process was repeated for six independent observations (n= 5) for each tissue to get statistically significant data on average (Grishagin, 2015).

Statistical Analysis: Conventional methods were used for calculation of means and SEM. Statistically differences among exposed groups were evaluated by Student's t-test. Data analysis was carried out using the GraphPad Prism software, La Jolla, CA, USA.

RESULTS AND DISCUSSION

To determine the harmful effect of CaC_2, our experimental model Swiss albino male mice (*Mus musculus*) were divided into four different groups and chronically exposed with 3gm, 5gm, 7gm and 9gm of CaC_2/day. After 40 days of exposure the percentage of survivability visibly altered (Fig. 1a). Survivability bar of the 3gm CaC_2 exposed group of animals decreased slightly and the change seemed non-significant with respect to the control group of animals ($p<0.05$). Animal survivability further sloped down gradually in 5gm and 7gm consecutively and significantly spiked in 9gm CaC_2 exposed animals.

As the route of exposure was through inhalation, the whole-body weight and targeted organ i.e., lung weight were estimated from respective groups of exposed animals. Values indicated that both the parameters have been increased in 3gm and 5gm of CaC_2 exposed group with respect to control, whereas, in 7gm the body weight drastically declined and organ weight increased (Fig. 1b). This sequence was reflected in the ratio-metric analysis of organ weight and body weight (Fig. 1c) where 3gm and 5gm CaC_2/day exposed groups depicted increasing patterns and 7gm showed a sharp spike.

The trend of alteration in body weight, organ weight exactly reversed in 9gm of exposed group and the organ weight-body weight ratio decreased. Considering the visible changes of body and organ weight, cell count was performed by calculating cell number in the 1mm x 1mm area of the histological section of lung tissue. Data were

obtained from six independent experiments (n=5) for each tissue to get statistically significant data on average. Result showed that cell number slightly increased in 3gm and further increased in 5gm CaC_2/day exposed group. Value suggested significant increase in cell count in 7gm and 9gm of CaC_2 exposed group with respect to control group of animals (Fig. 1d).

Survivability analysis was measured and represented as survivability percentage against control mice in 3gm, 5gm, 7gm and 9gm CaC_2 exposed group (a). Body weights and organ (lung) weights of the animals of control and four CaC_2 exposed groups were measured in gm (b) and ratio-metric analysis of organ (lung) weights and body weights were demonstrated (c). Number of cells at lung collected from control, 3gm, 5gm, 7gm and 9gm CaC_2 exposed group, were represented in cell count ($1X1mm^2$) (d). Data were expressed as mean$\pm$SD and were obtained from six independent experiments (n=5). NS, *$p<0.05$ vs Control. Con= Control.

The non-sequential variation in body weight-organ weight as well as cell number analysis clearly indicated pulmonary alterations which instigated us to estimate the activities of liver enzymes ALT (Alanine Aminotransferase) and AST (Aspartate Aminotransferase) as markers of body specific stability in mice. In both the observations activity levels of enzymes gradually elevated in the four groups of animals viz. 3gm, 5gm, 7gm, 9gm with respect to control, showing an ascending staircase trend along with the course of CaC_2 exposure (Fig. 2a, 2b). Such a result being an obvious indicator of disrupted body homeostasis and level of ROS (Reactive Oxygen Species) was quantified which serves as a regulatory index of the same.

ROS content was found higher in 3gm of CaC_2 exposed group of animals than rest of the exposed groups. 5gm and 7gm showed visibly lower ROS compared to 3gm CaC_2 exposure. In 9gm it was moderately high yet considerably lower than 3gm, exhibiting a dose responsive non- linear pattern of oxidative stress in four exposed groups (Fig. 2c). Activity of widely known antioxidant enzymes SOD and catalase were determined to validate the variation of the said oxidative stress.

SOD was activated in 3gm CaC_2 exposure and catalase activity was maintained to some extent with respect to control. As SOD activity increased in 5gm compared to 3gm, evidently potent ROS was generated but the maintained catalase activity in 5gm might have a role in lowering the oxidative stress. SOD activity reached its highest peak in 7gm and catalase activity was sustained. This phenomenon implied that a higher amount of CaC_2 exposure gave rise to an extra amount of ROS which was converted into H_2O_2 by high SOD activity, followed by persistent catalase activity that turned the additional H_2O2 into H_2O and O_2. Activity of both SOD and catalase significantly decreased in 9gm insinuating the probable crumpling of the protective antioxidant system (Fig. 2d)

Liver stress specific bio-markers ALT (a), AST (b) were estimated in blood serum isolated from control and 3gm, 5gm, 7gm and 9gm CaC_2 exposed group of mice. Values were represented in IU/L. Level of reactive oxygen species was estimated by measuring DCF fluorescence (c) and was represented in RFU. Lysates were processed from lungs isolated from control, 3gm, 5gm, 7gm and 9gm CaC_2 exposed groups. Status of SOD and catalase activities (d) were measured and were represented in U/mg protein. Data were expressed as mean+SD and were obtained from six independent experiments (n=5). *$p<0.05$ vs Control. Con= Control.

Different levels of ROS are the determining factors of cell fate via inducing either cell survivability or apoptosis. That instigated us to check the balance between anti-apoptotic and pro-apoptotic proteins. In Fig. 3b Densitometric analysis of anti-apoptotic Bcl2 protein showed significant decrease in 3gm of exposed group with respect to control. Values increased in 5gm and further in 7gm and a significant fall was portrayed in 9gm. Parallely, band intensity analysis of pro-apoptotic Bax protein demonstrated sufficient increase in 3gm and consequent decrease in 5gm and significantly in 7gm while value again escalated in 9gm CaC_2 exposure. Chief regulator of apoptosis p53 nearly mirroring the data of Bax, elevated in 3gm and gradually decreased in 5gm and 7gm exposure. A visible rise in intensity was found at 9gm after scheduled exposure (Fig. 3b). Band intensity of executioner caspase, the caspase3 was augmented in 3gm and 9gm, on the other hand value was reduced in 5gm and further in 7gm with respect to 3gm of exposure (Fig. 3c).

Changes in pro-apoptotic and anti-apoptotic balance instigated us to estimate the percentage of apoptotic cells under chronic CaC_2 exposure. Data depicted that FITC positive cells increased in 3gm (both in early and late) while a decreasing trend was observed in 5gm. Value of early apoptosis was further reduced while the number of late apoptotic cells were increased in 7gm exposure. The 9gm CaC_2 exposed group again exhibited sufficiently increased early apoptotic cells with a non-significant change in late apoptotic group with respect to control (Fig. 3a).

Cell cycle analysis indicated increased subG1 phase, reduced G1 and S as well as a proficient increase in G2/M in 3gm signifying increased cell death, cell proliferation and G2/M arrest. 5gm represented reduced subG1 to some extent, increased G1 and sustained S as well as reducing trend in G2/M arrest. 7gm depicted further reduced subG1, significant increase in G1, reduction in S and G2/M arrest indicating a trend towards cell survivability. 9gm indicated significant increase in subG1 with a decreasing trend in G1 and S as well as a trend towards increase in G2/M arrest representing cell death (Fig. 3d).

Apoptotic cell percentage were estimated from the pulmonary cells isolated from control, 3gm, 5gm, 7gm, 9gm CaC_2 exposed group (a). Bcl2, Bax and p53 expression (b), as well as caspase3 activity were measured in whole cell lysate of lung isolated from control and all of the four CaC_2 exposed groups of mice (c). Densitometric analysis of target specific bands were represented in AU. The cell percentage in different stages of cell cycle were estimated

in control and CaC_2 exposed groups of mice (d). Data were expressed as mean+SD and were obtained from six independent experiments (n=5). NS, *p<0.05 vs Control. Con= Control.

Countless reports have shown that technical grade CaC_2 contains traces of arsenic, phosphorus and other impurities. Assessment of CaC_2 treated mangoes via inductively coupled plasma mass spectrometry has also found traces of heavy metals like lead, chromium, cadmium (Hassan et al., 2019). The adverse effects of these impurities cause both acute and chronic conditions when ingested with CaC_2 contaminated food. Mostly in developing countries, due to lack of awareness, CaC_2 is used by many food vendors to boil eggs, soften the beans and to keep the food warm at the time of selling.

Previous in vivo studies suggest that oral intake of CaC_2 induces an array of abnormalities like derangement of hematopoiesis and organ toxicity (Ouma et al., 2022), disruption in reproductive system in both male and female mice (Bafor et al., 2019), hematological alterations (Appah et al., 2019), alteration in plasma electrolyte concentration and kidney function (Ugbeni and Alagbaoso, 2023). Compared to the effect of ingestion, toxicity through inhalation is more of a silent killer to the internal system. Through inhalation the toxicant directly reaches the lungs and hampers the normal gaseous exchange leading to accumulation of CO_2 and oxygen deprivation. As a result, blood pH falls which reduces tissue specific metabolic rate, also alters peripheral homeostasis. A study on the effects of chronic exposure of crude acetylene on white rabbits showed a significant increase in SOD activity heart, ALT and AST activities in serum while catalase activity was suppressed in heart, liver and kidney (Okolie et al., 2005). In this context our study dealt with the effect of CaC_2 exposure through respiration and therefore the primary affected organ was obviously lungs.

Activities of ALT and AST were altered under CaC_2 exposure which indicated unsteady body homeostasis. This finding led to the estimation of oxidative stress in targeted organ, lungs, as the chosen route of exposure was inhalation. The outcome showed that the modification of ROS happened in four CaC_2 exposed groups of animals in a dose dependent manner. Such variation of generated ROS probably was corroborated by the estimated status of SOD and Catalase activities.

CaC_2 exposure activated the SOD in the 3gm exposed group and further increased in 5gm. A balanced catalase activity was maintained in both the said exposed groups which perhaps helped in lowering the ROS generated under exposure. Activity status of SOD was expressed its peak value in 7gm and lowest value in 9gm of CaC_2/day exposed group. On the other hand, catalase increased in 7gm and visibly decreased in 9gm. Elevation of SOD and sustained activities of catalase possibly mitigated the additional ROS generated from increasing doses of CaC_2 exposure. This mechanism perhaps shielded the harmful effects of exposure up to a certain level and collapsed in the extreme condition. Excess cellular levels of ROS damages cell organelles which can lead to activation of programmed cell death. Moderate ROS contributes to the control of cell proliferation and differentiation (Perillo et al., 2020). In this study, alterations in the antioxidant system might lead to development of a stressed environment which is generally associated with the change in apoptotic balance shifting under experimental condition.

Here enhancement of cell death in lowest dose (3gm/day) of CaC_2 exposure and reduction of cell death in two median doses (5gm/day and 7gm/day) likely indicated the pro- to anti-apoptotic shifting of cellular fate. Further, the highest dose of exposure (9gm/day) indicated the augmentation of cell death due to another shifting of anti- to pro-apoptotic balance. Alteration in cell death probably reflected in organ weight since organ weight/body weight ratio was enhanced in 5gm and most effectively in 7gm CaC_2/day exposed group. More over increase in cell death perhaps helped in restoration of organ weight, as organ weight/body weight ratio in the 9gm CaC_2/day exposed group nearly returned to the control group of animals.

Compared to apoptotic status, the cell counts are almost maintained in 3gm and 5gm CaC_2 exposed groups with respect to control. Even though the early onset of apoptosis in both the cases were visibly significant, the accumulation of inflammatory cells under exposure probably played a role in this maintenance. In the 7gm of exposed group, low percentage of apoptotic cells indicated the way of direction towards cell survival and cell count were found high as it might be expected. Apoptotic cell percentage increased in 9gm but cell count also notably augmented. This was probably due to the accumulation of a higher number of inflammatory cells under a higher extent of exposure.

CONCLUSION

A number of physio-biochemical parameters were altered under CaC_2 exposure, but survivability of exposed animals was the most noticeable factor. In spite of high ROS and augmented cell death, survival rate in 3gm was highest amongst all exposed groups, perhaps due to 3gm being a considerably low and endurable dose of exposure. In 5 and 7 gm exposed groups, lower apoptosis probably indicated retention of damaged cells which might have resulted in low survival capacity of said groups. Higher rate of apoptosis in 9 gm possibly pointed towards elimination of exposure affected cells, thus increasing the survivability of mentioned group.

Author's Contribution: KDC and PC were responsible for conceptualization and designing of the study. SB and PG were responsible for model development, planning of experiments and data collection. KDC, SB, PG and DP were responsible for result analysis and interpretation. All authors equally contributed in literature research, manuscript preparation, editing and review.

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Statement of Ethics: This study was approved by the Institutional Animal Ethics Committee (IAEC), Rammohan College.

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Role of Mycorrhiza Colonization in Phosphorus Deficiency in Tomato Seedlings Affected by Different levels of Mycorrhiza Species

Randa S. Bairum[1*], Abdel Moneim E. Sulieman[2*], Mahdi A. A[3], Abdelbagi Mohamedelnour[4], Safa A. Sherfi[5], Hassan B. Elamin[6], Bandar Aloufi[2], Nujud Almuzaini[2] and Zakaria A. Salih[7]

[1]Department of Crop Protection - Faculty of Agriculture - Nile Valley University Atbara, Sudan
[2]Department of Biology, College of Science, University of Hail, Hail, Saudi Arabia
[3]Department of botany and agricultural biotechnology - Faculty of Agriculture, University of Khartoum Sudan
[4]Department of plant production and processing technology-Faculty of Agricultural Production and Processing Technology, International University of Africa
[5]Department Health Information Technology -Medical Sterilization Applied College- Sudan King Abdel Aziz University, Jedda Saudi Arabia
[6]Department of Biotechnology- National Research Center Sudan
[7]Department of Research and Training, Research and Training Station, King Faisal University, Al-Ahsa , Saudi Arabia

ABSTRACT

Most terrestrial plants respond to colonization by symbiotic mycorrhizal fungi, and these fungi have various benefits to their hosts under different stress conditions, especially phosphorus (P) limitation. A pot experiment was conducted to determine how tomato seedlings were affected by varying levels of mycorrhiza species under phosphorus deficiency conditions. A block experiment based on a completely randomized design was conducted in pots. Seeds of the tomato (*Solanum lycopersicum L.*) cultivar Beeli were inoculations with two species of arbuscular mycorrhizal (AM), namely; *Glomus mosseae* (MG), locally-isolated mycorrhizal spores (ML), *Fusarium oxysporum f.* sp. *lycopersici* (*FOL1*) pathogen, the myco-parasitic fungus (*Trichoderma harzianum*) (T_h) and effective microorganisms (EM@TM) at two different time 6 and 13 weeks.

KEY WORDS: ARBUSCULAR MYCORRHIZAL, PHOSPHORUS DEFICIENCY, TOMATO SEEDLINGS, FUSARIUM OXYSPORUM F. SP. LYCOPERSICI.

INTRODUCTION

Tomato (*Solanum lycopersicum L.*), with an annual production of 160 million tons, is one of the world's leading vegetables used in raw and processed forms (almost 40 million tons of tomatoes annually).Tomatoes are subject to many pests and diseases from the time of emergence to harvest. Among these, diseases incited by *Fusarium* are responsible for significant reductions in tomato quality and yield every year. Because the impact of tomato diseases cannot be predicted from one year to the next, certain precautions must be taken yearly to ensure maximum fruit production with minimum Fusarium wilt occurrence. Charoenporn et al. (2010) believe that *fusarium* wilt is one of the most severe tomato diseases worldwide. This disease is caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.), leading to severe economic losses Snyder and Hansen, (1940). It becomes one of the most prevalent and damaging diseases wherever tomatoes are grown intensively because the pathogen can persist indefinitely in infested soils (Agrios, 1997). Tomato is a critical horticultural crop that provides a wide range of necessary nutrients for human health (Imane, 2020).

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Terrestrial fungi can adopt different life strategies to exploit nutrient sources. They grow as saprotrophs on simple or complex organic substrates, or they can establish a nutritional relationship with higher plants, either biotrophs or necrotrophs. Mycorrhizal associations are the most critical mutualistic biotrophic interactions. Over 80% of vascular flowering plants can enter symbiotic associations with arbuscular mycorrhizal (AM) fungi. The fungi that form these associations are members of the zygomycetes, and the current classification places them all into one order: Glomales. They strictly depend on their host plant to complete their life cycle, whereas other mycorrhizal fungi, such as ericoid fungi, can be grown in pure culture.

The AM association is a relatively nonspecific, highly compatible, long-lasting mutuality from which both partners derive benefit. The plant supplies the fungus with carbon, on which it is entirely dependent. The fungal contribution is more complex. Although it is clear that the fungi assist the plant with acquiring phosphate and other mineral nutrients from the soil, AM fungi also may influence the plant's resistance to invading pathogens. In addition to its ecological significance, the association also may have applications in agriculture. This is particularly important because mycorrhizae link soil and plant and can improve plant nutrition efficiency and soil conservation.

The interaction begins when fungal hyphae arising from spores in the soil and adjacent colonized roots or hyphae contact the root surface. Here, they differentiate to form appressoria and penetrate the root. Appressoria formation is one of the first morphological signs of recognition between the plant and the fungus. Once inside the root, the fungus may grow inter- and intracellularly throughout the cortex, but AM fungi do not invade the vascular or the meristematic regions. The types of internal structures that develop depend on the plant/fungal combination and may include intracellular differentiated hyphae called arbuscules and intracellular coils.

Biotrophic fungi usually are thought to penetrate host tissues mechanically. It has been calculated that high pressure can be generated by appressoria of *Magnaporte grisea* (a nonmycorrhizal fungus) at the penetration point. This mechanical pressure allows the fungus to perforate the host wall by forming a penetration peg. Some wall components, such as melanin, play an essential role in increasing hydrostatic pressure since they trap solutes within the appressoria, causing water to be absorbed because of the increasing osmotic gradient.

There is an urgent need for environment-friendly management techniques, such as using arbuscular mycorrhizal fungi (AMF) to enhance crop productivity. AMF are commonly known as bio-fertilizers. Moreover, many believe that the inoculation of AMF provides tolerance to host plants against various stressful situations like heat, salinity, drought, metals, and extreme temperatures. AMF may assist host plants in the up-regulation of tolerance mechanisms and prevent the down-regulation of critical metabolic pathways. AMF, being natural root symbionts, provide essential plant inorganic nutrients to host plants, thereby improving growth and yield under unstressed and stressed regimes. The role of AMF as a bio-fertilizer can strengthen plants' adaptability to changing environments. Thus, further research on AMF-mediated crop quality and productivity promotion is needed. The present study provides comprehensive, up-to-date knowledge on AMF and its influence on host plants at various growth stages, their advantages and applications, and consequently, the importance of the relationships of different plant nutrients with AMF, (Naheeda et al.2019).

MATERIAL AND METHODS

Site description: The study site was the Demonstration Farm of the at the Faculty of Agriculture, University of Khartoum, at Shambat (15º 32´ N; 32º 32´E), Sudan.

Source of tomato cultivars: Tomato(*Solanum lycopersicum* L.)cultivar: Seeds of the cultivars were obtained from Shambat Research Station, Agricultural Research Corporation (ARC).

Isolation of the pathogen: *F. oxysporium lycopersici* was isolated from naturally diseased tomato plants exhibiting typical symptoms of wilt disease. Infected parts of the plants will be excised with a sterile scalpel and was surface sterilized with 30% (w/w) NaOCl for 2 min. Sterilized pieces was washed with sterile water and cut into small pieces (1cm length) and transferred on to antibiotic amended PDA plates. Plateswas incubated at room temperature for 48 h and mycelial growth from the infected stem pieces will be transferred into new PDA plates. After incubation for 5 days, single spores were isolated and cultured on new PDA plates. The pathogen was identified based on the characteristics described by Booth (1977). Koch's postulates were demonstrated for the pathogen and confirmed as the causal agent of wilt of the tomato plant.

Pathogen inocula: The pathogen inocula was produced on PDA plates. The plates was inocubated with an agar plug (5 mm in diameter) containing actively growing *F. oxysoprium* mycelium and incubated under fluorescence for 10 days at room temperature. Spores were washed from the plates with sterile distilled water and the concentration was adjusted to 106 spores mL-1 with a haemocytometer.

Disease assessment: Disease Severity (DS) and Disease Incidence (DI) of *Fusarium* wilt and *Rhizoctonia* were assessed 21 days after inoculation for each treatment.

Disease severity was estimated visually by assessing brown rot on the root and hypocotyls using a rating scale of 0-5

$$\text{Disease Severity} = \frac{\sum ab \times 100}{AK}$$

Where:
a = No. of diseased plants having the same degree of infection
b = Degree of infection
A = Total no. of examined plants
K = Highest degree of infection

Analysis of growth and yield parameters: Three plants of each treatment were harvested, three weeks after inoculation with the pathogens.

2-washed under running water to remove soil particles and evaluated for the following growth parameters:
a) shoot fresh and dry weight (g). b) root fresh and dry weight (g)
c) shoot and root length (cm).
d) leaf area (cm2).

Dry weights were recorded after drying the samples at 80^0 C for 48 h. in a hot air oven until constant weight.

AM inocula: In this investigation, a mixture of formulated AM (Multi-VAM) spores kindly provided to units of *Glomus mosseae* in suspension form (1×10^6 unit L^{-1} in concentration). In addition to this *Glomus mosseae* inoculum, another treatment consisting of locally-isolated mycorrhizal spores was used. These spores were isolated from Shambat soil.

Planting and growth conditions: Pots were filled with disinfested soil at the rate of 2.5 kg pot^{-1}; clay: sand (2:1, v/v). Five healthy seeds of tomato will be sown in each pot. Half of the pots will receive AM inocula as a suspension twice, in the tomato seed bed at the beginning and as a soil drench 14 days after the sowing at dilution of 5 ml L^{-1} water (El-Haddad et al., 2004). All plants were fertilized with phosphorus, all pots was kept outdoor under natural conditions and watered when necessary.

After four weeks of AM inoculation, five milliliters of spore's suspension (*F. oxysporium*) were applied by pipette just below the collar region around the hypocotyls of each plant. Ten pots were treated only with plain water to serve as a control. Ten other pots were used as replicates for each treatment. Fifteen plants from each treatment were harvested after three weeks after inoculation with the pathogen for different analyses. The treatments to be applied in this study has been summarized as follows: Control (CNM), AM (CM), Pathogen (PNM) and Pathogen + AM (PM). The trials were conducted twice, and the experiments have been arranged in a completely randomized block design.

Concentration of phosphorus: The phosphorus content was determined according to Chapman and Pratt (1961). Shoots dried samples (2g) of tomato seedlings were ground. The samples were ashed in a furnace at 550C for 3 hr. Ten mL of 0.5N HCl were added to the ashed sample and heated gently on a hot plate. The ashed solution was quantitatively transferred to a 100 mL volumetric flask, made up to volume with deionized distilled water, and shaken. 2 ml from the ashed solution were taken in a conical flask (50), then 10 ml ammonium vanadates were added, and the volume was completed to 50 ml with distilled water. After 30 min, the absorbance at 470 nm was then determined by dosing a UV spectrophotometer (JENWAY 6305UV/vis).

Statistical analysis: Analysis of data variance was performed using Statistics Package 8.

Staining and estimation of mycorrhizal root colonization: Fixed roots were rinsed repeatedly in tap water; cut into small segments (0.5 to 1cm) and bleached once in a KOH (10%) solution for 45 min at 90^0 C, darker roots will be bathed in alkaline hydrogen peroxide for 20 min (Kormanik and McGraw, 1982). Thereafter, the roots were washed with tap water three times and stained with 0.05% trypan blue in lactophenol for 15 min at 900 C (Phillips and Hayman,1970). The excessive stain was washed with tap water. Fifty randomly selected stained root pieces were mounted on slides in lactoglycerol and examined microscopically for estimation of mycorrhizal root colonization according to the method of Trouvelot et al. (1986).

Experiment design: A block experiment based on a completely randomized design was carried out with three replications. The experimental setup consisted of three treatments: a control group with no inoculums and two groups inoculated with mycorrhizal fungus (*Glomus mosseae*) (M_G) and locally isolated mycorrhizal spores (M_L), respectively. These treatments were organized as the first and second variables. The two species of AM fungi (M_G) and (ML) used in this study were isolated from Shambat soil by using the "wet sieving and decanting" technique of Hayman (1982). *Fusarium oxysporum* f. sp. *lycopersici* (*FOL1*) pathogen was isolated from naturally diseased tomato plants exhibiting typical symptoms of wilt disease and identified based on the characteristics described by Booth (1977).

The Effective Microorganisms (EM™) was obtained in solution form from Moroug Co. Khartoum North, an agent of EmroJapan.com.

The Myco-parasitic fungus (*Trichoderma harzianum*) (T_h) Rifai was isolated from commercial BIOCONT from Organicsolutions-me.com by plating and preparing in PDA.

The tomato seeds were sterilized with 0.05% sodium hypo-chloride for 45 minutes before sowing. Seeds were germinated into sterilized soils (clay: sand) at a ratio of (2:1, v/v) in plastic pots (15x15 cm). Half of the pots received AM inoculum as a suspension twice: in the tomato seedbed at the beginning and as a soil drench 14 days after sowing, added dilution of 5 ml L^{-1} water of 6.12×10^3spores /mL (El-Haddad et al. 2004). Three weeks after sowing, seedlings were inoculated with the pathogen (*FOL1*) by injecting spores suspension near the roots at the rate of 5 mL (containing approx. 5.13×10^4spores $/mL^{-1}$) per seedling.

Control plants were sown with nonmycorrhizal and no injection. Plants were grown under natural photoperiods, temperature, and relative humidity conditions, fertilized with phosphorus super phosphate P_2O_5 (5.23×10^{-4} g/pot), and watered every other day. At 21 days, the percentage of disease incidence (DI) and disease severity (DS) were recorded (Filion et al., 2003). At 6 and 13 weeks after transplanting, the parameters include leaf area (cm2), shoots and roots length (cm), and ground fresh and dry weights (g) of seedlings were measured after harvesting. The new weights (g) were measured before drying at 80°C for 28 h

in a hot air oven until constant weight led to the dry matter weights (g).

The extent of colonization of tomato roots by arbuscular mycorrhizal (AM) fungi was assessed at two distinct time points (6 and 13 weeks) after harvest. Roots were rinsed repeatedly in tap water, cut into small segments (0.5 to 1cm), and bleached once in a KOH solution (10%) for 45 min at 90^0 C and stained in 0.05% lactic acid– glycerol–Trypan Blue (Phillips & Hayman, 1970). Darker roots were bathed in alkaline hydrogen peroxide for 20 minutes (Kormanik & McGraw, 1982). Fifty randomly selected stained root pieces were mounted on slides in lactoglycerol and examined microscopically for estimation of mycorrhizal root colonization following the method of Biermann and Linderman (1981).

Figure 1: *F. oxysporium* lycopersici inocula

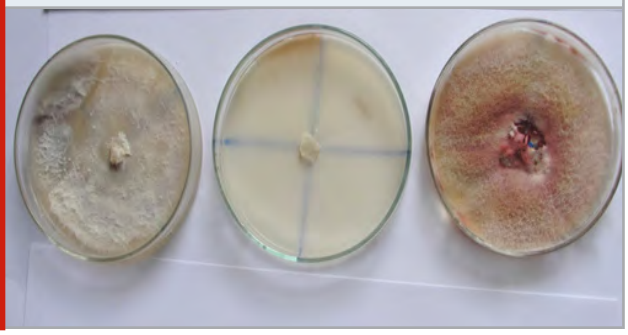

Table 1. Disease Incidence (DI%) and Disease Severity (DS%) on Tomato var. Beeli at 6th and 13th Weeks from Transplanting.

Treatments	Six weeks	Thirteen weeks	
	DI (%)	DI (%)	DS (%)
Control	57.50^{b}	7.05^{c}	12.80^{a}
FOL1	77.50^{a}	53.25^{a}	40.78^{a}
ML	50.00^{b}	20.25^{bc}	12.78^{a}
F_{OL1} + ML	65.00^{ab}	23.00^{b}	22.50^{a}
F_{OL1} + ML + MG	50.00^{b}	29.00^{b}	34.78^{a}
F_{OL1} + Th	62.50^{ab}	25.50^{b}	18.05^{a}
F_{OL1} + EM	62.50^{ab}	18.25^{bc}	31.25^{a}
± SEM	9.06	7.06	13.43

RESULTS AND DISCUSSION

Tomato variety was experimentally infected with *FOL1*, with a disease incidence of 77.50%, significantly dropping 13 weeks from transplanting (Table 1). However, all inoculation treatments, including the local mycorrhiza (ML), dual mycorrhizal inoculation (M_G), *T. harzianum* (T_h), and the Effective Microorganisms (EM^{TM}), were effective in reducing both disease incidence and severity at 6 and 13 weeks (Table 1). However, no clear superiority could be attributed to any of these treatments as their effects on disease amelioration showed apparent fluctuations between 6 and 13 weeks. It is noted that disease incidence and severity in the non-infected plants at 13 weeks were lower than in all infected plants, albeit vaccination with any of the above treatments.

Mean separations were performed, and differences at $P < 0.05$

Table 2.a. shows the effects of the treatments on some above-ground growth parameters of Tomato var. Beeli after six weeks from transplanting. In the F_{OL1}-infected plants, all treatments (M_L, M_L+M_G, *T. harzianum*, or EM^{TM}) resulted in significantly ($p \leq 0.05$) higher leaf areas, which were even more significant than those of uninfected plants (controls and those receiving the local mycorrhiza). The highest benefit was due to the combination of mycorrhizae and EM^{TM}. A substantial increase was recorded in the 3.47% -16.52% range in the leaf area. A noticeable decrease in the leaf area in the 25.00% - 4.00% range was also obtained.

As for shoot length, all inoculation treatments could reduce the effects due to *FOL1,* except for plants inoculated with *T. harzianum,* and were all better than control plants except in the case of *T. harzianum* and EMTM. (Table 2. a). Results of the investigations in which Tomato var. Beeli and *FOL1* (alone on shoot length) showed a substantial increase in the 2.38% -7.14% range. A noticeable decrease in the shoot length range of 15.20% - 0.25% was also obtained. In varying degrees, the various inoculation treatments improved shoot fresh weight in the *FOL1*-infected plants. Still, these treatments did not yield new weights higher than control plants except for plants treated with the dual mycorrhizal inoculum or *T. harzianum*.

On the other hand, none of the inoculated treatments could produce shoot dry weights higher than those of the control plants. They could not improve the infected plants' dry weight except those receiving the dual mycorrhizal inoculation or *T. harzianum*. A basic increase in shoot fresh weight was recorded in the 20.71% -46.44% range. Also, there was a noticeable decrease in the shoot new weight in the range of 28.10% - 14.79%, whereas in shoot dry weight, the reduction was 11.11%. A noticeable decrease in the shoot dry weight in the 41.66% - 19.44% range was also obtained (Table 2. a).

Mean separations were performed, and differences at $P < 0.05$

At 13 weeks, all inoculated treatments produced leaf area greater than in the *FOL1*-inoculated treatment, and all, except for the combined mycorrhizal treatment, were more significant than the control treatment. A substantial increase in leaf area was recorded in the range of 9.81% -30.47%. A noticeable decrease in the leaf area in the 15.76 % - 9.55% range was also obtained (Table 2.b).

Shoot fresh weights in the *FOL1*-infected plants were also improved, to varying extents, by all inoculation treatments, which produced higher new weights than in the control plants except for plants receiving the dual mycorrhizal

inoculation. Likewise, all inoculation treatments improved the infected plants' shoot dry weight and shot length. Still, two of them (the two mycorrhizal inoculations) could not surpass control plants in these two parameters. A basic increase in shoot fresh weight was recorded in the 17.64 % -99.60% range. Also, a noticeable decrease in the shoot new weight in the range of 39.12% - 31.96%, whereas in shoot dry weight was recorded in the range of 10.08% -54.62%. A noticeable decrease in the shoot dry weight in the range of 56.30% - 13.44% was also obtained (Table 2.b).

Table 2.a: The effects of the treatments on some above-ground growth parameters of Tomato var. Beeli, at six weeks from Transplanting

Treatments	Leaf area (cm)	Change (%)	Shoot length (cm)	Change (%)	Shoot fresh wt.(g)	Change (%)	Shoot dry wt. (g)	Change (%)
Control	94.83^{d}	0.00	27.30^{c}	0.00	3.38^{d}	0.00	0.36^{ab}	0.00
F_{OLI}	71.00^{g}	-25.00	23.15^{f}	-15.20	2.43^{f}	-28.10	0.26^{abc}	-27.77
M_L	98.13^{c}	+3 .47	27.95^{b}	+2.38	2.68^{e}	-20.71	0.24^{bc}	-33.33
F_{OLI}+ M_L	76.13^{f}	-19.71	25.08^{d}	-8.13	4.08^{c}	+20.71	0.21^{c}	-41.66
F_{OLI}+ M_L + M_G	110.50^{a}	+ 16.52	29.25^{a}	+7.14	2.88^{e}	-14.79	0.29^{abc}	-19.44
F_{OLI}+ T_h	90.95^{e}	-4.09	27.23^{c}	-0.25	4.95^{a}	+46.44	0.40^{a}	+11.11
F_{OLI}+ EM	100.25^{b}	+5.71	24.20^{e}	-11.35	4.56^{b}	+34.91	0.21^{c}	-41.66
±SEM	0.58		0.15		0.10		0.07	

Mean separations were performed, and differences at $P < 0.05$

Table 2.b. The effects of the treatments on some above-ground growth parameters of Tomato var. Beeli, at 13 weeks from Transplanting

Treatments	Leaf area (cm)	Change (%)	Shoot length (cm)	Change (%)	Shoot fresh wt.(g)	Change (%)	Shoot dry wt. (g)	Change (%)
Control	100.17^{e}	0.00	33.05^{d}	0.00	5.10^{e}	0.00	1.19^{d}	0.00
FOL1	84.38^{g}	-15.76	30.10^{g}	-8.92	3.10^{g}	-39.12	0.52^{g}	-56.30
ML	110.00^{d}	+9.81	32.03^{e}	-3.08	6.00^{d}	+17.64	1.03^{e}	-13.44
FOL1+ ML	120.27^{b}	+20.06	34.08^{c}	+3.11	9.03^{b}	+77.05	1.31^{c}	+10.08
FOL1+ ML + MG	90.60^{f}	-9.55	31.05^{f}	-6.05	3.47^{f}	-31.96	0.61^{f}	-48.73
FOL1+ Th	115.45^{c}	+15.25	35.00^{b}	+5.90	8.46^{c}	+66.88	1.58^{b}	+32.77
FOL1+ EM	130.70^{a}	+30.47	36.00^{a}	+8.92	10.18^{a}	+99.60	1.84^{a}	+54.62
±SEM	0.96		0.05		1.22		1.02	

Mean separations were performed, and differences at $P < 0.05$

Mean separations were performed, and differences at $P < 0.05$

At six weeks, the data in Table 3. showed that the root length was also more significant in the inoculated *FOL1*-infected plants than in the un-inoculated treatments, whether infected or not. The longest roots were recorded in infected plants inoculated with *T. harzianum*, followed by those treated with EM™, whereby a substantial increase was recorded in the range of 26.31% -117.93%. A noticeable decrease in the root length of 16.56% was also obtained. Root fresh weight was also significantly ($p \leq 0.05$) higher in the inoculated

treatments than in the FOL1-infected plants, except for those receiving the mycorrhizal combination, than in the uninfected plants. Similarly, all inoculation treatments resulted in higher root dry weight than in *FOL1*-infected plants, all of which, except those receiving M_L or EM™, were superior to the uninfected plants. A substantial increase in root fresh weight was recorded in the 28.12% - 46.87% range. There was also a noticeable decrease in the root new range of 34.37% - 6.25%, whereas root dry weight was recorded in the 2.12%- 63.82% range. A noticeable decrease in the root dry weight in the 59.57% - 20.22% range was also obtained (Table 3.a).

At 13 weeks, the root length of the infected plants was improved by all inoculation treatments, which produced roots longer than in control plants except for plants receiving the mycorrhizal injections (Table 3.b). Likewise, all treatments had higher root fresh weights, which were more elevated than the FOL1-infected plants.

Figure 2: Experiment design

Table 3.a: The effects of the treatments on some root growth parameters of Tomato var. Beeli, at six weeks from Transplanting

Treatments	Root length (cm)	Change (%)	Root fresh wt. (g)	Change (%)	Root dry wt. (mg)	Change (%)
Control	5.13^{e}	0.00	0.32^{c}	0.00	9.4^{bc}	0.00
FOL1	4.28^{f}	-16.56	0.21^{e}	-34.37	3.8^{d}	-59.57
ML	5.28^{e}	+29.23	0.41^{b}	+28.12	7.5^{c}	-20.22
FOL1+ ML	6.48^{d}	+26.31	0.26^{d}	-18.75	5.3^{d}	-43.61
FOL1+ ML + MG	7.53^{c}	+46.78	0.30^{cd}	-6.25	9.7^{b}	+3.19
FOL1+ Th	11.18^{a}	+117.93	0.47^{a}	+46.87	9.6^{bc}	+2.12
FOL1+ EM	10.10^{b}	96.88	0.41^{b}	+28.12	15.4^{a}	+63.82
±SEM	0.33		0.03		9.69	

Mean separations were performed, and differences at $P < 0.05$

Table 3.b: The effects of the treatments on some root growth parameters of Tomato var. Beeli, at 13 weeks from Transplanting

Treatments	Root length (cm)	Change (%)	Root fresh wt. (g)	Change (%)	Root dry wt. (mg)	Change (%)
Control	8.88^{ab}	0.00	0.83^{d}	0.00	0.31^{c}	0.00
FOL1	5.17^{d}	-41.77	0.42^{g}	-49.39	0.07^{f}	-77.41
ML	9.08^{ab}	+2.25	0.91^{c}	+9.63	0.13^{e}	-58.06
FOL1+ ML	8.05^{bc}	-9.34	1.23^{a}	+48.19	0.41^{b}	+32.25
FOL1+ ML + MG	7.13^{c}	-19.70	0.50^{f}	-39.75	0.09^{f}	-70.96
FOL1+ Th	9.85^{a}	+10.92	1.01^{b}	+21.68	0.45^{a}	+45.16
FOL1+ EM	9.08^{ab}	+2.25	0.64^{e}	-22.89	0.23^{d}	-25.80
±SEM	0.58		0.02		9.43	

Mean separations were performed, and differences at $P < 0.05$

Still, only three (those co-inoculated with the local mycorrhiza, with *T. harzianum*, and those inoculated with the local mycorrhiza without *FOL1* infection) were better than control plants. Similarly, all produced higher root dry weights than the FOL1-infected plants, but only two (those receiving the local mycorrhiza and those inoculated with *T.*

harzianum) were better than control plants. A substantial increase in root fresh weight was recorded in the 9.63 % -48.19% range. The degree of colonization of tomato roots by arbuscular mycorrhizal (AM) fungi was evaluated at two specific time intervals (6 and 13 weeks) following harvest. A noticeable decrease in the root dry weight in the range of (77.44% - 25.00%) was also obtained (Table 3.b). Table (4) shows root colonization by the mycorrhizal fungi in Tomato var. Beeli at six weeks from transplanting. Very low colonization occurred in FOL1-infected plants that did not receive vaccination and in control plants. Highest root colonization was recorded in FOL1-infected plants co-inoculated with EMTM, followed by plants co-inoculated with the local mycorrhiza or T. harzianum, and then uninfected plants inoculated with the local mycorrhiza. A basic increase in root colonization was recorded in the range of (120.00% -265.00 %).

Table 4. Mycorrhizal Colonization percentage of Tomato var. Beeli affected by different levels of mycorrhiza species in 6th Week from Transplanting

Treatments	Root colonization (%)
Control	20.0
F_{OL1}	44.0
ML	59.0
F_{OL1} + ML	62.0
F_{OL1} + ML + MG	60.0
F_{OL1} + Th	62.0
F_{OL1} + EM	73.0

Mean separations were performed, and differences at $P < 0.05$

Table 5: Phosphorus Content (ppm) of Tomato var. Beeli is affected by different levels of mycorrhiza species at the 6th and 13th Weeks from Transplanting

Treatments	Six weeks (ppm)	Thirteen weeks (ppm)
Control	0.94^{cd}	0.45^{c}
F_{OL1}	1.33^{cd}	0.71^{bc}
ML	2.28^{b}	0.91^{ab}
F_{OL1} + ML	3.65^{a}	1.17^{a}
F_{OL1} + ML + MG	1.70^{bc}	0.54^{c}
F_{OL1} + Th	1.00^{cd}	0.66^{bc}
F_{OL1} + EM	0.74^{d}	0.44^{c}
±SEM	0.39	0.16

Mean separations were performed, and differences at $P < 0.05$

Table 5 shows that plant phosphorus content (ppm) of FOL1-pathogenic plants was much higher than in un-pathogenic (control) plants at 6 and 13 weeks, and the values recorded at 13 weeks were much lower than those recorded at six weeks. However, this content was significantly improved by injection of the pathogenized plants with either the local mycorrhiza or both mycorrhizae at six weeks and the local mycorrhiza at 13 weeks. In the un-pathogenic plants, although the phosphorus content was much lower than in FOL1-pathogenic plants, vaccination with the local mycorrhiza significantly improved the phosphorus content at 6 and 13 weeks.

Fungi are involved in a wide range of intimate symbiotic associations with other organisms, and it would be no exaggeration to say that they have shaped the history of life on land. In several cases, fungi and their partners have become so intimately dependent on one another that they have lost the ability to live separately. In many cases, it is possible to cultivate fungi in laboratory media. Still, they are, in effect, ecologically obligate symbionts or parasites because they seldom grow as free-living organisms in nature (Deacon, 2006).

Tomatoes are susceptible to a wide variety of fungal pathogens and other diseases. The most important are those caused by pathogenic fungi (Apodaca-Sánchez et al., 2002; Carrillo-Facio et al., 2003). Tomato's most crucial fungal disease is *Fusarium* wilt, caused by *Fusarium oxysporum* Schlechtend, f. sp. *lycopersici* (Sacc.) W.C.Snyder and H.N. Hans (FOL) can reduce yield by up to 60% and affect fruit quality (Agrios, 2004). *Fusarium* wilt of Tomato is a hypoplastic disease that causes reduced development and is similar in most respects to vascular fusarioses of various other plants (Fierro-Coronado et al., 2013).

In the present study, the plants invariably exhibited symptoms of vascular wilt, formation of yellow patches, and wilting after 21 days of pathogenization. Plant growth parameters were, to a large extent, negatively affected, and the degrees of disease incidence and severity were great. Nevertheless, injection of these *Fusarium*-pathogenic plants with the two arbuscular mycorrhizal fungi (*Glomus mosseae* or the locally isolated mycorrhiza, or their combination) improved Tomato plant growth. It succeeded in alleviating the harmful effects of vaccination with the *Fusarium* isolates alone, suggesting that these fungi can be an effective and environmentally sustainable biological treatment to counter these ill effects and increase Tomato plants' growth. These AM fungi could be inoculated during transplantation or in tomato nurseries (Utkhede, 2006).

Considered a contribution to protect Tomato plants (*Solanum lycopersicum L.*) against wilt disease, this study highlighted physiological and biochemical aspects of Tomato AMF mycorrhization, which could enhance resistance through the improvement of growth (nutrient supply) and the activation of the defense mechanisms of host plants against of F. o. f. sp. *lycopersici*, the causal agent of wilt disease. Nutrients such as N, P, K, Mg, and Ca are required by tomato crops at the right time and in the correct

quantity for sound production and yield (Olaniyi JO and Ajibola AT, 2008).

The various microorganisms present in the rhizosphere arbuscular mycorrhizal (AM) fungi are of great value in promoting the growth and yield of plants (Siddiqui & Mahmood, 1998). Colonization by AM fungi has been studied to increase the absorption of minerals, particularly immobile nutrients, from the soil by the host.

The present study revealed that inoculation with AM fungi singly or in a combination enhanced plant growth in all the varieties of Tomato plants tested. This considerable increase in growth and dry weight could be attributed to the increase in intake of nutrients such as phosphorus, nitrogen, potassium, and other micronutrients by the co-inoculated AM fungi. Previous studies in different vegetable crops (Artursson et al., 2006) have reported significantly increased shoot and root dry weights when inoculated with AM fungi.

CONCLUSION

The present study has demonstrated the beneficial role that can be played by some biocontrol agents (the two mycorrhizal fungi *Glomus mosseae* and the local mycorrhizal isolate, the fungus *T. harzianum* and the commercial microbial blend EMTM) in increasing plant resistance against the infection with FOL. Different physical and physiological mechanisms have been shown to play a role in plant protection by these treatments, namely, improved plant nutrition and growth.

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Biotechnological Communication

Synergetic Impact of Sodium Azide and Ethyl Methane Sulfonate Treatment on SSR Marker-Based Assessment of Okra Seedling Genetic Purity

Mayur S. Dhole, Ashwinikumar B. Kshirsagar* Ashok A. Shinde and Kundan N. More
Department of Plant Breeding & Molecular Genetics, Institute of Biosciences & Technology,
MGM University, Chhatrapati Sambhajinagar 431003, Maharashtra State India

ABSTRACT

In this comprehensive study, the synergetic impact of sodium azide and EMS mutagenic treatments on 'Parbhani Kranti' okra seeds and plants were rigorously examined. The study employed okra seeds in a CRD, implementing five treatments (T0 to T4) with varied concentrations of Sodium Azide and EMS. The primary goal was to assess genetic purity, covering morphological parameters, DUS tests; Physiological observations included chlorophyll, RW content and molecular analysis by using SSR Marker. Morphological parameters, including shoot, root, seedling length, and leaf area were adversely affected, with T4 showing the most stunted growth. Flowering delay and altered fruit characteristics were also observed, correlating with mutagen concentrations. The findings of this research reveal that seeds subjected to mutagenic treatments exhibited significantly reduced germination rates when compared to the control. The most substantial reduction, at 70%, were observed in seeds treated (T4) and was detrimentally influenced by these mutagenic interventions. The increase chlorophyll (mean 1.47) and RWC (mean 47.06) complex plant responses to mutagenic elements. Potential adaptive or tolerance mechanisms, including increased chlorophyll production and water retention, may be activated to mitigate stress. Genetic purity assessments exposed variations in DNA content and quality across different treatments. Among the two SSR marker differentiated allelic size of 260 and 300 bp. The allelic sizes are specific to the seed and pollen of the hybrid. These findings hold implications for developing novel okra cultivars and ensuring genetic purity in hybrid seed production, contributing to agricultural sustainability.

KEY WORDS: CHEMICAL MUTAGEN, MUTATION BREEDING, OKRA, POLYMERASE CHAIN REACTION, SSR PRIMERS.

INTRODUCTION

Okra (*Abelmoschus esculentus Moench*) is a warm-season annual herbaceous vegetable crop from the Malvaceae family, believed to originate in South Africa. It holds significant economic importance due to its widespread consumption as a cooked vegetable (Mishra et al., 2015). Characterized by its self-pollinating nature, it grows as a shrub, reaching 1 to 2 meters in height, with large leaves (20 to 40 cm in length, exhibiting 3-7 lobes) and round, large, grey seeds (Akinyele and Osekita, 2006). It thrives during the summer months, dependent on specific climatic factors like light, temperature, and water availability. An average rainfall of 665 mm is crucial for its productive cycle, though germination can be challenging (Tiwari et al., 1998), (More et al., 2023).

Beyond its agricultural significance, okra is a rich source of essential vitamins and minerals, including carbohydrates, iron, potassium, and magnesium. Okra seeds are notable for their high protein content (15%-26%) and edible oil content exceeding 14%. The entire okra plant, including its mucilaginous fruit, is edible and can be consumed fresh, cooked, added to salads or soups, or preserved. The seeds are also rich in unsaturated fatty acids, water, proteins, carbohydrates, and fiber (Kumar et al., 2013). Additionally, the seeds contain high levels of polysaccharides, contributing to their medicinal properties, and are even used to create a coffee substitute known as "chocolate de los negros" (Mishra et al., 2015).

Okra is cultivated globally, covering approximately 1.117 million hectares and yielding 8.706 million tonnes, with an average productivity of 7.8 tonnes per hectare. India is the

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largest contributor to okra production, accounting for 72% of the world's output. States like Gujarat, Maharashtra, Andhra Pradesh, and others are primary regions for okra cultivation (Gandhi, 2014). India and Nigeria are leading producers, while minor producers include Pakistan, Ghana, Egypt, and more. Okra serves as a profitable crop for many developing countries, generating income and employment opportunities, and is predominantly exported to the United States (Adiger et al., 2011). Mutation breeding accelerates genetic variations and the emergence of new species by exposing seeds to chemicals, radiation, or enzymes. Mutation breeding is swifter than traditional methods and introduces both qualitative and quantitative variations (Gupta, 2019).

Physical mutagens involve electromagnetic and particle radiation, while chemical mutagens include alkylating agents and azides. Chemical mutagens are milder and easier to apply, inducing both loss-of-function and gain-of-function mutations, such as herbicide tolerance (Oladosu, et al., 2015). In the future, DNA profiling may be employed for genotype characterization, providing valuable data for plant breeding. Molecular markers, especially Simple Sequence Repeats (SSR), are pivotal in genetic assessment, as they can detect genetic variations at the DNA level, facilitating the evaluation of genetic purity (Gandhi et al., 2014).

Mutagenic treatments, including sodium azide and EMS, induce genetic variations in plants. By subjecting okra seeds to these treatments, a higher mutation rate is achieved, leading to a broader spectrum of genetic variations (Pharmawati et.al, 2018). In combination with SSR markers, this approach allows for precise identification of genetic variations in okra seedlings, facilitating the selection of genetically pure lines and adherence to DUS guidelines (Jamshed et al., 2016).

These findings have significant implications for the development of novel okra cultivars and the crucial task of safeguarding genetic purity in hybrid seed production.

MATERIAL AND METHODS

The experimental site for this study was the Institute of Biosciences and Technology in Chhatrapati Sambhajinagar. Okra seeds obtained from VNKV, Parbhani, were used as the plant material. The research design employed was a Completely Randomized Design (CRD). The primary focus of the study was 'Parbhani Kranti,' a variety of *Abelmoschus esculentus* known for its resistance to yellow vein mosaic viruses. The research involved five treatments labeled as T_0, T_1, T_2, T_3, and T_4, with each treatment having different concentrations of Sodium Azide and EMS. The treatments were as follows: T_0 (Control), T_1 (0.10 + 0.05 mM), T_2 (0.20 + 0.10 mM), T_3 (0.30 + 0.15 mM), and T_4 (0.40 + 0.20 mM). The primary goal of this research was to evaluate genetic purity and related characteristics in 'Parbhani Kranti' okra seedlings. The study encompassed both morphological and physiological observations. Morphological observations included monitoring various parameters such as seed weight, germination percentage (calculated as Germination % = (Number of germinated seeds / Total tested seeds) x 100), seedling vigor (determined by shoot-root length difference x Germination %), and measurements of shoot and root length at 10, 20, and 30 days post-germination.

Table 1. Synergistic Impact of Sodium Azide and EMS on Morphological Traits of Okra

Treatment	Morphological Parameters of Okra Seedling (30 Days)				
	Germination %	Seedling length (cm)	Seed vigour index	Leaf area (cm^2)	Survival %
T0	97	26	25.22	50.5	96
T1	80.33	23	21	46	78
T2	78.66	20.66	16.25	44	75
T3	73.33	19	13.93	42	70
T4	70.33	18.33	12.89	34	67
Mean	79.93	21.39	17.86	43.3	77.2
SE	7.39	7.13	7.29	7.22	7.43
CD (0.05)	11.04	2.77	2.65	4.32	10.2

The length of seedlings and leaf area was also recorded. Additionally, survival percentage was calculated based on the comparison of surviving plants to the initial number of plants, and a Distinctness, Uniformity, and Stability (DUS) test was conducted on 30 plants divided into three replications. In terms of physiological observations, the study involved the estimation of chlorophyll content using the Arnon Method with a spectrophotometer. Although it was mentioned that relative water content was measured, the specific method used was not provided.The study also included DNA isolation, which was performed using a Plant Genomic DNA Mini-preparation kit. This process involved grinding in liquid nitrogen, buffer addition, heating, centrifugation, RNAse A treatment to remove RNA, ethanol precipitation, and air drying.

DNA quality was assessed through 0.8% agarose gel electrophoresis, and DNA quantification was carried out using a spectrophotometer and SoftMax Software. Purity was assessed using the the L_1/L^2 ratio. SSR Marker screening was performed via PCR amplification with 18 microsatellite markers linked to okra traits, while correlation detection examined the relationships between SSR banding patterns and fruit length, strength, and yield quality. The PCR program included denaturation, annealing, extension steps, and a final extension at 72°C.

The experimental data were analyzed using statistical methods, with the experiment conducted under a Completely Randomized Design (CRD) (Panse and Sukhmate, et al. 1968). This study represents a comprehensive investigation into the genetic purity and related traits of 'Parbhani Kranti' okra seedlings, encompassing both morphological and physiological aspects.

RESULTS AND DISCUSSION

The study aimed to evaluate the Synergetic impact s of chemical mutagens (Sodium azide and EMS) on the morphological, physiological, and molecular characteristics of Okra genotypes. The outcomes of the study are summarized as follows. One-factor ANOVA was used to analyze the morphological and molecular parameters of Okra seedlings at 30 days after sowing (DAS). The data for various treatments are presented in (Table 1). Germination percentage is a crucial parameter for assessing mutagenic Synergetic impact s on plants. It serves as a reliable indicator of mutagen impact on seed viability.

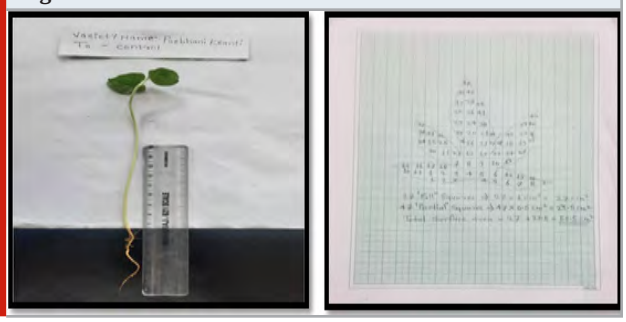
The control group (T_0) exhibited the highest germination percentage at 97%. Germination percentages decreased as the dose of mutagen increased. The lowest germination percentage was observed in T_4 (0.40 + 0.20) treated with sodium azide + EMS, with a germination rate of 70%. Treatment groups T_1, T_2, T_3, and T_4 had germination percentages of 80%, 78%, 73%, and 70%, respectively are shown in (figure 1). The decline in germination can be attributed to the inhibitory Synergetic impact s of mutagens on essential physiological processes, including enzymatic activity, hormonal balance, and mitotic activity crucial for seed germination. The data indicates a linear relationship between mutagen dose (sodium azide + EMS) and reduced seed germination. These findings demonstrate that increased mutagen concentrations negatively affect seed germination, highlighting the importance of carefully controlling mutagenic treatments for crop improvement. In the next sections of the discussion, the results from other morphological, physiological, and molecular parameters were discussed, providing a comprehensive understanding of the impact of chemical mutagens on Okra genotypes (Table 1).

These findings demonstrate that increased mutagen concentrations negatively affect seed germination, highlighting the importance of carefully controlling mutagenic treatments for crop improvement. In the next sections of the discussion, the results from other morphological, physiological, and molecular parameters were discussed, providing a comprehensive understanding of the impact of chemical mutagens on Okra genotypes. In this study, the morphological characteristics of the "Parbhani Kranti" okra variety were examined after subjecting it to treatments involving Sodium azide + EMS. Observations were recorded 30 days after germination (Table 1).

Figure 1: Germinated seeds.

Figure 2: Seedling length.

Figure 3: Leaf area.



The treated plants showed a decrease in shoot length compared to the control. The most significant reduction was observed in seeds treated with T4 (0.40+0.20) of sodium azide and EMS, with a shoot length of 11 cm compared to the control's 15 cm. Similarly, root length decreased slightly in treated plants, with the lowest root length in seeds treated with T4. The seedling length was affected by the treatment, with a decrease as the treatment concentrations increased are shown in (figure 2) (Table 1).

Leaf area was also reduced in treated plants, and, interestingly, a higher treatment concentration led to both

reduced leaf area and an increase in the number of leaves. These results indicate that Sodium azide + EMS treatment has a significant impact on the morphological characteristics of the okra variety, with reduced shoot length, root length, seedling length, and leaf area compared to the control. This suggests that the mutagenic treatment affected the plant's growth and developments are shown in (figure 3) (Table 1).

The table 2 shows the impact of mutagen treatments on okra plants, specifically in terms of flowering, fruiting, and fruit characteristics. Plants treated with higher mutagen concentrations flowered earlier and had more flowers. For example, T_4 had the highest number of flowers at 11, while T_1 had the lowest with 6 flowers.

Figure 4: TSynergetic impact of mutation on Fruit Height.

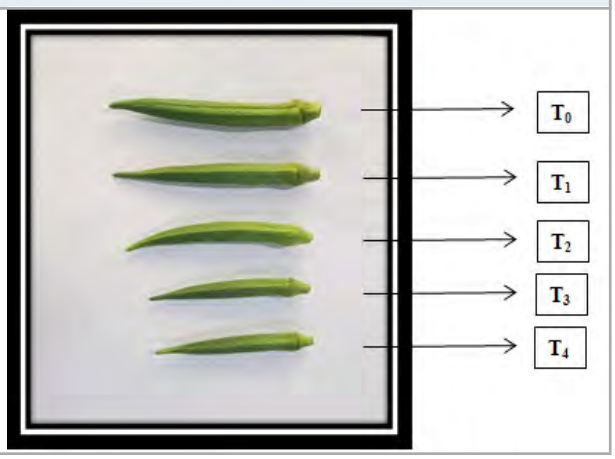


Figure 5: Agorse gel electrophoresis.

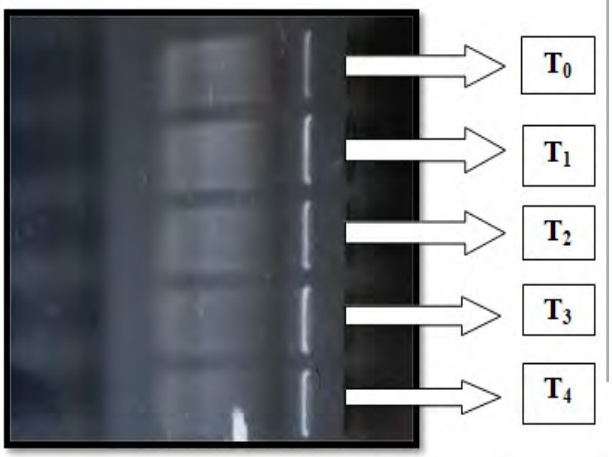


Table 2. Synergetic impact s on the Number of Flowers and Number of Fruits in Okra Plants

Treatments (T)	Sowing to Flowering Duration (Days)	No. of Flowers	Flowering to Fruiting Duration (Days)	No. of Fruits	Fruit			
					Colour	Shape	Height (cm)	Weight (gm)
T0	45	10	20	3	Green	Straight	8.3	10.03
T1	48	6	23	2	Green	Curved	7.2	8.35
T2	50	8	21	2	Green	Curved	6.9	7.17
T3	49	9	19	3	Green	Straight	5.5	5.09
T4	44	11	19	4	Green	Straight	4.8	4.53
Mean	47.2	8.8	20.4	2.8	Green	Straight	6.54	7.03
SE	7.39	2.38	7.32	1.43			4.67	5.87
CD (0.05)	11.04	6.89	11.01	5.39			12.02	11.8

(Treatment T0 ; Number of seed /fruit 62; T1 Number of seed /fruit 57; T2 Number of seed /fruit 53; T3 Number of seed /fruit 50 ; T4 Number of seed /fruit 51)

Higher mutagen concentrations led to more fruits, with T4 producing the most (4 fruits), and the control (T_0) having 3 fruits. Fruits from higher concentration treatments (T_3 and T_4) were shorter and lighter. For example, T_4 had the shortest fruits at 4.8 cm and the lowest weight at 4.53 gm. In contrast, the lower concentration (T_1) produced longer and heavier fruits. The number of seeds per fruit varied among treatments, with T_0 having the most seeds (62), and T_3 having the fewest (50) shown in (figure 4) (Table 2). Mutagen concentration affects flowering, fruiting, and fruit characteristics in okra. Higher concentrations lead to more flowers and fruits but result in shorter, lighter fruits with fewer seeds, while lower concentrations produce larger fruits with more seeds.

DUS Characteristics: The profile of 36 Okra cultivars was established using a set of morphological characteristics as prescribed in the DUS test guidelines for Okra. These characters were employed to establish distinctiveness, uniformity, and stability of each cultivar, forming the basis

for cultivar protection. The Parbhani Kranti cultivar was utilized, and chemical mutagenic treatment was applied. Subsequently, the resultant variety was used for the analysis of DUS characteristics. Out of these 29 DUS characters, the states in which each character was found are presented in (Table 3).

Table 3. Distinguishing Unique Characteristics of Cultivated Plant Varieties (DUS)

Sr.No.	Characters	States	Variety
1	Stem: Colour	Green	Green
2	Stem: Intensity of green colour	Light	Light
3	Leaf blade: Depth of lobbing	Medium	Medium
4	Stem: Number of nodes at first flowering	Many (>8)	Many (>8)
5	Flowering: Time	Late (>45 days)	Late (>45 days)
6	Leaf blade: Width (cm)	Large (>25)	Large (>25)
7	Leaf blade: Serration of margin	Strong	Strong
8	Leaf blade: Colour between veins	Green	Green
9	Leaf blade: Intensity of colour between veins	Medium	Medium
10	Vein: Colour	Light Green	Light Green
11	Petiole: Length (cm)	Medium (20-30)	Medium (20-30)
12	Flower: Petal colour	Yellow	Yellow
13	Flower: Petal base colour	Both side	Both side
14	Flower: Length (cm)	Large (>5)	Large (>5)
15	Fruit: Colour	Green	Green
16	Fruit: Length (cm) at marketable stage	Medium	Medium
17	Fruit: Diameter	Medium (1.0-.5)	Medium (1.0-.5)
18	Fruit: Surface between ridges	Flat	Flat
19	Fruit: Pubescence	Medium	Medium
20	Fruit: Constriction of basal part	Strong	Strong
21	Fruit: Shape of apex	Acute	Acute
22	Fruit: Number of locules	<6	<6
23	Plant: Number of branches	Many (>4)	Many (>4)
24	Stem: Diameter	Large (>1.5)	Large (>1.5)
25	Plant: Height	Tall (>120)	Tall (>120)
26	Fruit: Length of Physiologically mature fruit	Long (>15)	Long (>15)
27	Seed Colour	Green	Green
28	Seed: Hairiness	Present	Present

In the physiological characterization of the okra varieties, two significant parameters, namely chlorophyll content and relative water content (RWC), were evaluated (Table 4). Chlorophyll A, chlorophyll B, and total chlorophyll (chlorophyll ab) were determined and recorded. Among the treatments, the highest chlorophyll content was observed in the highest treatment of EMS + Sodium azide (T_4), with a value of 1.90, surpassing other treatments and the control (Table 4).

The relative water content (RWC) was calculated using a specific formula, and observations were recorded. The highest RWC was observed in the EMS + Sodium azide T_4 treatment, with a value of 51.81%, surpassing other treatments and the control. This indicates that the RWC was significantly influenced by the mutagenic treatment. The complete data and calculations for RWC should be provided in your research, as it's crucial for understanding the physiological response of the okra seedlings to the treatments. The formula used to calculate RWC and the specific values for each treatment should be included (Table 4).

The Relative Water Content (RWC) was calculated for different treatments of okra seedlings. The results show variations in RWC among the treatments. The highest RWC was observed in the EMS + Sodium azide T_4 treatment, with a value of 51.81%. In contrast, the control (T_0) had an RWC of 63.36%. The significant differences in RWC among the treatments suggest that the mutagenic treatment

had an impact on the water content of the seedlings. This physiological parameter provides insights into the response of okra seedlings to the mutagenic agents. The complete data and the statistical calculations are essential for a comprehensive understanding of the physiological changes induced by these treatments.

The formula used for calculating RWC and the specific values for each treatment should be presented in your research for clarity and reference (Table 4). Significant variations were observed in almost all morphological traits, including seed germination, shoot length, and the number of leaves, as well as physiological traits such as chlorophyll content and relative water content in wheat plants exposed to salt and drought stress. These traits exhibited substantial changes, particularly in the varieties NIAW-917, NIAW-295, and MACS-6222, when compared to NIAW-301 and NIAW-3170. Notably, the relative water content was highest in NIAW-3170, measuring at 78.03%, but it decreased to 71.25% under the influence of water and salt stress and chlorophyll content experienced a significant reduction under stress conditions (More et al., 2023).

This DNA isolation process is essential for subsequent molecular analyses and genetic studies, ensuring that high-quality DNA is obtained for accurate and reliable results. The genomic DNA extracted from the okra seedlings was quantified using a spectrophotometer. The quantification was performed to ensure the quality and quantity of the DNA samples shown in (figure 5). The data are presented in the following (Table 5). The values of the DNA concentration for all treatments (T_0, T_1, T_2, T_3, and T_4), ranging from 72.5 ng/µl to 94.0 ng/µl. The highest DNA concentration is found in T_0 (94), while the lowest concentration is observed in T_3 (72.5). This quantification data is vital for subsequent research and ensures the availability of suitable DNA samples for further study (Table 5), (figure5).

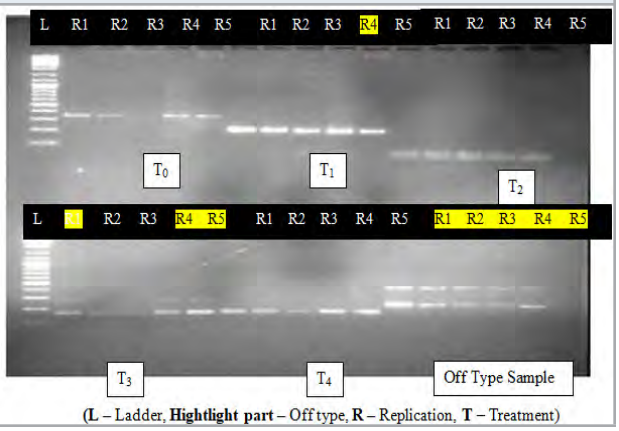
Table 4. Synergetic impact of EMS and Sodium Azide on Physiological Parameters.

Treatments (T)	Chlorophyll Content			Relative Water content (%)
	Chl. A	Chl. B	Chl. AB	
T0	0.96	0.98	1.95	63.36
T1	0.53	0.54	1.18	43.30
T2	0.49	0.48	1.06	35.76
T3	0.62	0.69	1.25	41.09
T4	0.90	0.97	1.90	51.81
Mean	0.70	0.73	1.47	47.06
SE	1.93	2.34	3.73	7.393
CD (0.05)	8.45	8.04	9.69	11.046

Table 5. Synergistic Influence of EMS and Sodium Azide on DNA Quantification

Treatments (T)	Concentration (ng/µl)
T_0	94
T_1	85.5
T_2	74
T_3	72.5
T_4	86.5
Mean	82.5
C.D(0.5)	1.52
S.E	12.74

Figure 6: Combine effect of sodium azide and EMS on genetic purity of Okra.



(**L** – Ladder, **Hightlight part** – Off type, **R** – Replication, **T** – Treatment)

PCR Analysis: The assessment of genetic purity is vital in maintaining seed quality during okra hybrid seed production and ensured the genetic purity of the seeds; SSR (Simple Sequence Repeat) markers are used to differentiate the allelic size of 260 and 300 base pairs. These markers help confirm the specificity of allelic size in the seeds and their association with both the seed and pollen parents of the hybrid. Following chemical mutagen treatment, the DNA sequence may be altered, leading to changes in the SSR marker patterns. The results indicate that the first marker is linked only to the control (T_0) and not to the DNA bands

of other treatments. In certain conditions, the marker may be linked to other markers, resulting in the appearance of double bands on the agarose gel.

To assess the purity of Parbhani Kranti seeds, bulked samples from both rows and columns were analyzed on the agarose gel. The amplification of both allelic sizes (260 and 300 bp) was expected. The presence of double bands in specific coordinates indicated potential contamination. To confirm the identity of off-type individuals, leaf samples from seedlings at these coordinates were analyzed. The results showed that the allele size of seedlings at these coordinates was similar to the off-type coordinate. Therefore, based on the presence of double bands, it was possible to confirm them as off-type seedlings (Table 6).

Table 6. Assessment of Genetic Purity and Total Off-Type Percentage in Cultivated Plant Varieties

Treatment (T)	Off Type Plants	Percent of off Type (%)	Genetic Purity (%)
T_0	0	0	100
T_1	1	10%	90%
T_2	0	0%	100%
T_3	3	30%	70%
T_4	0	0%	100%

In the agarose gel, when five contaminated samples were added, they also exhibited double bands, indicating that they were off-type seedlings. Based on the presence of double bands in three coordinates, it was inferred that these samples were also contaminated and classified as off-type seedlings. To test the genetic purity of Parbhani Kranti subjected to chemical mutagenic treatments, they were analyzed for single or double bands. The presence of double bands, as seen in T1-R4 and T3-R1, R4, R5 coordinates, indicated the presence of off-type seedlings. Based on these results, the percentage of genetic purity was calculated and is presented in the following (Table 6) shown in (figure 6). The table provides the percentage of off-type plants and the corresponding genetic purity for each treatment. These results are crucial for evaluating the genetic purity of Parbhani Kranti and identifying off-type seedlings.

The study investigated the synergetic impact of Sodium azide and EMS mutagen treatment on the Parbhani Kranti okra variety. Several key findings emerged: Germination rates decreased as mutagen concentration increased. Control (T_0) had the highest germination rate at 97%, while T_4 (highest mutagen concentration) had the lowest at 70%. Shoot and root length decreased in mutagen-treated plants. The lowest values were observed in T_4 table 6.

Higher mutagen concentrations led to increased flower and fruit numbers. However, these fruits were smaller and lighter in weight. Chlorophyll content increased with higher mutagen concentrations. RWC also increased with mutagen concentration.

Genomic DNA was successfully isolated, and DNA concentrations varied among treatments.SSR markers revealed contamination in some treatments, impacting genetic purity. T_3 showed the highest off-type percentage. These findings highlight the complex Synergetic impact of mutagen treatment on okra, with implications for breeding and genomic diversity preservation in this crop. Assessment of genetic purity of seed is crucial for maintaining the seed quality in okra hybrid seed production. Among the two SSR marker differentiated with allelic size of 260 and 300 base pairs respectively.

Thus ensuring that allelic size are specific to the seed and Pollen parent of the hybrid. The increase in chlorophyll content and relative water content with an increase in mutagenic elements concentrations suggests a complex response in plants. The exposure seed of the plant species one hour, the interpretation is as - Plants may be responding to the presence of mutagenic elements by activating adaptive or tolerance mechanisms. This could involve an increased production of chlorophyll, which is crucial for photosynthesis, and an enhancement of water retention to cope with potential stress. The increase in chlorophyll and water content could be a stress response.

In some cases, exposure to mutagenic elements might trigger stress responses that include physiological changes in an attempt to mitigate the negative Synergetic impact s of the stressor. Plants strive to maintain internal balance or homeostasis. The increase in chlorophyll content and water retention may be a part of the plant's efforts to regulate internal conditions and sustain essential processes. Mutagenic elements have the potential to induce genetic changes. The observed increase in chlorophyll and water content may be a result of genetic alterations that influence the plant's physiological characteristics. Different plant species may respond differently to mutagenic elements, and even within a species, individual plants may exhibit varying responses.

Therefore, the interpretation of these changes would be more accurate if specific details about the plant species and the mutagenic elements involved are known. It's important to consider that an increase in chlorophyll and water content may not necessarily indicate a positive or beneficial outcome for the plant. It could be an adaptive response to stress, and prolonged exposure to mutagenic elements might have negative consequences on overall plant health and productivity. Further research and analysis are needed to understand the specific mechanisms at play and the long-term Synergetic impact on the plants in question.

CONCLUSION

In summary, exploration into the Synergetic impact of sodium azide and EMS mutagens on Parbhani Kranti okra illuminates the intricate dynamics between genetic alterations and plant response. Higher concentrations of mutagens emerged as catalysts for reduced germination rates and distinct shifts in plant morphology, exposing the vulnerability of okra to mutagenic stress.

The chlorophyll and water content hints at a genetic recalibration, suggesting an adaptive mechanism triggered by environmental stress. This dual emphasis on mutagenic impacts and genetic purity enhances our understanding of the intricate dance between genetic modification and sustainable crop breeding, echoing a resounding imperative for precision and foresight in the pursuit of resilient and high-yielding okra

Conflict of Interest: Author declare no conflicts of interests to disclose.

Authors contribution: Formulation of the research concept, experimental design, provision of experimental materials, execution of field and laboratory experiments, data collection, analysis, interpretation of data, and manuscript preparation.

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On the Diversity and Taxonomic Evaluation of Wood-Decaying Fungi from Ajanta Forest Caves, Maharashtra, India

Vijay Udhav Gore[1] and Vasant Pandit Mali[2]
[1]Department of Biology, Shiveshwar Junior College Takli (A), Taluka Kannad, District Aurangabad India
[2]J. Watumull Sadhubella Girls College, Ulhasnagar Dist. Thane India

ABSTRACT
An the present investigation, 89 specimens of wood-decaying fungi were collected between the viewpoint and Ajanta cave of the famous ecotourist spot of Ajanta forest, research was carried out during year 2014 to 2021. Specimens were identified according to macroscopic characteristics on site and also in microscopic details in the laboratory. As far as we know we are reporting for the first time the diversity of wood-decaying fungi from Ajanta Forest, which consists of 28 genera and 39 species. Among these 35 species were annual and 4 species were perennial. Most dominating to rarely observed genus were *Trametes* Fr (5 species) followed by *Leucocoprinus* Pat (3 species), *Auricularia* Bull (2 species), *Favolus* Fr (2 species), *Hypoxylon* Bull (2 species), *Lentinus* Fr (2 species), *Phellinus* Quél (2 species), whereas *Cellulariella* Zmitr. & Malysheva, *Cerrena* S.F. Gray, *Daedaleopsis* Schroet, *Daldinia* Ces. & de Not, *Duportella* Pat, *Earliella* Murrill, *Flavodon* Ryvarden, *Funalia* Pat *Ganoderma* P. Karst, *Hexagonia* Pollini, *Lopharia* Kalchbr. & MacOwan, *Phanerochaete* P. Karst, *Phlebiopsis* Julich, *Phylloporia* Murrill, *Pleurotus* (Fr.) P. Kumm, *Pseudofavolus* Pat, *Pycnoporus* P. Karst, *Scytinostroma* Donk, *Schizophyllum* Rr, *Truncospora* Pilát, and *Xylaria* Hill ex Schrank represent single genera.

KEY WORDS: AJANTA CAVE, MACROSCOPIC, MICROSCOPIC, SPECIMENS,

INTRODUCTION

Ajanta forest is located in Sillod and Soygaon Tehsil Area of Aurangabad district in Maharashtra, India. The world famous Ajanta cave is situated at 20° 33' 8.56" N 75° 42' 1.57" E. Specimens were collected in between Ajanta caves and upper view point of Ajanta forest. Basidiocarp of macro-fungi are formed only when ecological conditions are favorable, but their mycelia exist on humus, plant litter, and decaying wood for a long period. Macro-fungi fruiting on woody substratum are usually saprobes or pathogens causing root rot, butt rot, heart rot, and decay of wood, branches, and twigs. Studies of Wood-decaying fungi were initiated with the launch of studies in Indian fungi. The first Indian record of Wood-decaying fungi could be traced back to work (Klotzsch, 1832) in his paper Indian Polyporaceae. Later few Indian Polypores described by (Berkeley, 1839).

Bose was the first Indian mycologist to provide comprehensive account on wood-decaying fungi from Bengal (Bose, 1919a,b,c, and 1927a,b,c). He took special efforts to publish a book entitled "Genera of Indian Polypores" in which he was the first to describe the diversity and taxonomy of Indian Polypores (Sharma, 2000). Checklist of 256 species of Aphyllophorales fungi from Western-ghats of Maharashtra state have included 170 species from 10 poroid families and 86 species from 20 non-poroid families (Ranadive et. al 2011). 10 Genera and 13 species of gilled fungi collected from Pune and Western Ghats of Mahabaleshwar and Mulshi have also been described by Senthilarasu (2014). Similarly, 27 genera and 23 species of wood-rotting fungi from Asti-1 have been described by Mali, (2015). India represents about 20 species under genus Trametes from that, 5 species have been reported from Nanded and Parbhani Districts of Marathwada region of Maharashtra (Mali, 2016). 22 genera and 27 species of wood-decaying fungi have also been reported from Soygaon, District Aurangabad India, recently by Gore and Mali, (2023). As far as we know we are reporting for the first time the diversity of wood-decaying fungi from Ajanta Forest are3a caves, Aurangabad, India, which consists of 28 genera and 39 species. Among these 35 species are annual and 4 species are perennial.

MATERIAL AND METHODS

Wood-decaying fungi were collected 15 to 20 days after heavy rainfall month of July to November from year 2014-

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2021 from various region of Ajanta forest near Ajanta cave. The Basidiocarp of fungi is first photographed at the site then noted down morphological features by using a hand lens (20 X) dimension, color, shape, consistency, upper sterile surface, lower fertile surface, margin, context, tubes, and pores per mm in the field book and then specimens are dried naturally under sun light or using 200 watt bulb maintaining temperature 40-55° C. Microscopic observations were done by taking free hand thin section cutting of Basidiocarp with the help of sharp razor blades, stained and studied in 10 % KOH, Lactophenol, and Melzer's reagent under 40X and 100X Magnification (Olympus CX 41) in laboratory. Then specimens of macro-fungi were kept in brown paper packets as per international mycological herbarium guidelines according to date of collection, locality, host name, altitude, latitude, longitude, and classification of species. Naphthalene balls were placed in each herbarium packet to avoid insect attack.

RESULTS AND DISCUSSION

28 genera and 39 species of wood decaying fungi were identified according to morphological and microscopic character from collected 89 specimen are as follow (Table-1 and Photo plate-1).

***Auricularia mesenterica* (Dicks.) Pers:** Basidiocarp annual, resupinate to pileate, 0.7–27.2 × 0.5–10.1 cm, up to 0.3 cm thick, moist dependent, bracketed, soft jelly like. Pileus 0.5–3.4 × 0.3–0.5 cm, up to 0.3 cm thick, ear like, hairy, forming greyish white to brownish black. Lower fertile surface smooth to slightly wrinkled, purplish brown to coffee brown. Context very thin, jelly like when fresh. Hyphal system monomitic, generative hyphae 3–6 µm wide. Spores 8–13 × 5.5–6.5 µm, ovoid to reniform.

***Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García**: Basidiocarp annual, pileate, moist dependent, soft jelly like. Pileus 0.9–3.8 × 0.5–2.7 cm, up to 0.4 cm thick, ear like, attached with the help of short stalk like apparatus narrowly attached, velvety hairy, tuft of hairs forming greyish white to almost brownish black. Lower fertile surface smooth, purplish brown to coffee brown. Context jelly like when fresh, homogeneous. Hyphal system monomitic, generative hyphae 3–5 µm wide. Spores 14–16.5 × 5.5–7.5 µm, allantoid.

***Cellulariella acuta* (Berk.) Zmitr. & Malysheva:** Basidiocarp annual, pileate, sessile. Pileus 8.9–15.4 × 5.1–8.4 × 0.7–2.6 cm thick near base, semicircular. Upper surface sterile, concentrically zonate, sulcate, yellowish white to dark blonde. Lower fertile surface poroid to maize like 1–4 mm wide pores, yellowish white to pale yellow. Context up to 0.8 cm wide. Tubes up to 1.8 cm wide. Hyphal system tirmitic, generative hyphae 2–3 µm wide, skeletal hyphae up to 3.5–6.5 µm wide, binding hyphae 2.5–4 µm wide. Spores 5.5–7 × 2–3 µm, cylindrical.

***Cerrena caperata* (Berk.) Zmitr:** Basidiocarp annual, pileate, sessile. Pileus 1.3–4.2 × 1–2.4 × 0.2–0.4 cm, applanate, dimidiate. Upper sterile surface, tomentose, chocolate brown to grayish brown. Lower fertile surface poroid, 3–5 per mm, round to angular, cinnamon to deep chocolate-brown. Context 0.1–0.2 cm thick. Tubes 0.1–0.2 cm long, cinnamon brown. Hyphal system trimitic, generative hyphae 1–2 µm wide, skeletal hyphae 3–5 µm wide, binding hyphae 2–4 µm wide, Spores 6.5–9 × 2–3.5 µm, cylindrical.

***Daldinia concentrica* (Bolton) Ces. & De Not:** Basidiocarp annual, globose,1.6–3.8 × 1.5–3.3 × 1.1–2.4 cm, hemispherical, hard when fresh, brittle to charcoal like on drying, purple brown to brownish black. Fertile surface smooth, glabrous, composed of single layer spore bearing flask like organ, perithecia1–2 mm wide, tubular to lanceolate, slightly papillate ostioles. Context composed of alternating zonation and each zone represent seasonal growth. Perithecia 800–1100 × 300–500 µm, lanceolate, small, crowded in a single layer beneath the thin crust. Asci 200–260 × 7–12 µm, cylindrical, 8-spored. Spore 12–17 × 5–7 µm, elliptic-fusiform.

***Daedaleopsis confragosa* (Bolt : Fr.) Schroet:** Basidiocarp annual, pileate, sessile, dimidiate. Pileus 12.3–17.4 × 8.1–10.3 cm, up to 2.7 cm thick near base, semicircular, applanate, azonate to concentrically zonate, sulcate, yellowish white to dark blonde. Lower fertile surface poroid, lamellate to maize like up to 1mm wide pores, pastel yellow to buff. Context up to 2 cm wide. Tubes up to 0.7 cm wide. Hyphal system tirmitic, generative hyphae 2–4.5 µm wide, skeletal hyphae up to 4–6 µm wide, binding hyphae 3–4 µm wide. Spores 9–11 × 2–3 µm, cylindrical.

***Cerrena caperata* (Berk.) Zmitr:** Basidiocarp annual, pileate, sessile. Pileus 1.3–4.2 × 1–2.4 × 0.2–0.4 cm, applanate, dimidiate. Upper sterile surface, tomentose, chocolate brown to grayish brown. Lower fertile surface poroid, 3–5 per mm, round to angular, cinnamon to deep chocolate-brown. Context 0.1–0.2 cm thick. Tubes 0.1–0.2 cm long, cinnamon brown. Hyphal system trimitic, generative hyphae 1–2 µm wide, skeletal hyphae 3–5 µm wide, binding hyphae 2–4 µm wide, Spores 6.5–9 × 2–3.5 µm, cylindrical.

***Daldinia concentrica* (Bolton) Ces. & De Not:** Basidiocarp annual, globose,1.6–3.8 × 1.5–3.3 × 1.1–2.4 cm, hemispherical, hard when fresh, brittle to charcoal like on drying, purple brown to brownish black. Fertile surface smooth, glabrous, composed of single layer spore bearing flask like organ, perithecia1–2 mm wide, tubular to lanceolate, slightly papillate ostioles. Context composed of alternating zonation and each zone represent seasonal growth. Perithecia 800–1100 × 300–500 µm, lanceolate, small, crowded in a single layer beneath the thin crust. Asci 200–260 × 7–12 µm, cylindrical, 8-spored. Spore 12–17 × 5–7 µm, elliptic-fusiform.

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wide. Hyphal system tirmitic, generative hyphae 2–4.5 µm wide, skeletal hyphae up to 4–6 µm wide, binding hyphae 3–4 µm wide. Spores 9–11 × 2–3 µm, cylindrical.

***Duportella tristicula* (Berk. & Broome) Reinking:** Basidiocarp annual, crust-like or resupinate, 0.5–24.1 × 0.4–3.9 × 0.02–0.1 cm thick when young, initially arises as small velvety brown patches growing in all direction to form a large fruiting body, papery thin and brittle on drying, smooth, brownish grey dark brown Fertile surface smooth and shiny, velvety when young, dark brown to coffee brown. Context papery thin. Hyphal system dimitic, generative hyphae 2–3.5 µm wide, skeletal hypahe 2.5–4 µm wide, Spores 8.5–11 × 3–5 µm, ellipsoid.

***Earliella scabrosa* (Pers.) Gilb. & Ryvarden:** Basidiocarp annual, resupinate, effused reflexed to pileate, 0.5–14.9 × 0.4–8.7 × 0.2–0.6 cm. Pileus 0.5–6.9 × 0.3–3.4 × 0.2–0.6 cm, semicircular, light zonate, sulcate, glabrous, creamy white to reddish brown. Lower fertile surface poroid, 1–3 pores per mm angular to iripicoid yellowish grey. Context up to 0.2 cm thick, Tube up to 0.4 cm wide. Hyphal system trimitic, generative hyphae 2–3.5 µm wide, skeletal hyphae 3.5–5 µm wide, binding hyphae 3–5 µm wide. Spores 7–9 × 3–4 µm, cylindrical to oblong ellipsoid.

***Favolus grammocephalus* (Berk.) Imazeki:** Basidiocarp annual, pileate. Pileus 5.7–11.2 × 4.2–6.7 cm and up to 0.7 cm thick at the base, applanate, dimidiate to flabelliform, yellowish white to ochraceous to pale brown. Lower fertile surface poroid 3–4 per mm pores, round to angular, yellowish white to brownish yellow. Context 0.1–0.4 cm wide. Tubes 0.1–0.2 cm long. Stipe 0.3–0.6 × 0.3–0.5 cm, laterally attached. Hyphal system dimitic, generative hyphae 2.5–5 µm wide, skeleto-binding hyphae 4.5–7 µm wide, Spores 5–6 × 2–3 µm, ellipsoid.

***Favolus roseus* Lloyd:** Basidiocarp annual, pileate. Pileus 3.6–5.8 × 1.9–4.6 × 0.1–0.7 cm, semicircular, greyish golden yellow to yellowish brown. Lower surface fertile poroid, 1–2 per mm, hexagonal to pentagonal, pale yellow to orange yellow. Context up to 0.2 cm wide toward the base. Tube up to 0.5 cm wide. Stipe 0.1–0.5 × 0.3–0.4 cm, lateral to eccentric. Hyphal system dimitic, generative hyphae 2.5–5 µm wide, skeleto-binding hyphae 2.5–6 µm wide, Spores 8–12 × 3.1–5.1 µm, cylindrical.

***Flavodon flavus* (Klotzsch) Ryvarden:** Basidiocarp annual, resupinate to effused-reflexed 1.8–42.7 × 0.8–13.2 × 0.1–0.7 cm thick. Pileus 1.5–33.9 × 0.3–1.7 cm up to 0.7 cm thick, applanate, glabrous when matured, grayish yellow to olive grey. Lower fertile surface poroid, pores or lamellate or teeth 1–2 per mm, lemon yellow to ochraceous. Context up to 0.2 cm wide. Tubes up to 0.5 cm long, olive yellow. Hyphal system dimitic, generative hyphae 2.5–6 µm wide, skeletal hyphae 3.5–6 µm wide. Spores 5–7 × 2.5–4 µm, broadly ellipsoid.

***Funalia leonina* (Klotzsch) Pat:** Basidiocarp annual, pileate. Pileus 0.6–9.1 × 0.5–5.8 × 0.7–1.9 cm thick near the base, applanate, smooth, covered with stiff persistent and strigose hairs, yellow white to pale yellow to ochre orange Lower fertile surface poroid, pores 1–2 per mm, toothed, iripicoid to maize like, cream to orange brown. Context up to 1.2 cm thick. Tubes up to 0.6 cm wide. Hyphal system trimitic, generative hyphae 3.5–6 µm wide, skeletal hyphae 3–5 µm wide, binding hyphae 2.5–3.5 µm wide. Spores 11–14.5 × 3.5–5 µm cylindrical.

***Ganoderma mediosinense* J.D. Zhao:** Basidiocarp annual, pileate. Pileus 10.3–12.9 × 7.2–8.4 and up to 3.1 cm thick near stipe juncture, semicircular, glabrous. Upper surface often covered with a cocoa brown powder of deposited spores, faint reddish brown to dark reddish brown. Lower fertile surface poroid round, pores 3–4 per mm, cream when young to pale brown when old. Context up to 2.4 cm wide. Tubes up to 0.7 cm deep. Stipe 5.3–8.9 × 0.7–1.4 cm. Hyphal system trimitic, generative hyphae 1.5–3 µm wide, skeleton-binding hyphae 2–5.5 µm wide, binding hyphae 1.5–3 µm wide. Spores 9–11 × 5–6.5 µm, ovoid to ellipsoid.

***Hexagonia* sp. 1:** Basidiocarp annual, resupinate, effused-reflexed to pileate. Pileus 4.2–8.9 × 2.5–3.7 × 0.3–2 cm, semicircular, sterile, zonate, sulcate, glabrous, greyish brown to tobacco brown. Lower fertile surface poroid 1per mm wide, angular to hexagonal, teak brown to brownish grey. Context up to 0.7 cm wide. Tubes up to 1.3 cm long. Hyphal system trimitic, generative hyphae, 2–3.5 µm wide, skeletal hyphae 3.5–6.5 µm wide, binding hyphae 3–4 µm wide. Spores 5–7 × 3–4.5 µm, cylindrical.

***Hypoxylon haematostroma* Mont:** Basidiocarp annual, resupinate, 0.5–12.3 × 0.5–5.5 × 0.1–0.4 cm, hard. Fertile surface minutely papillate, cinnabar red to reddish brown. Context papery thin, homogenous, solid, dark brown. Perithecia long tubular 900–2300 × 200–600 µm. Ostioles are lower than stromatal surface. Asci 150–200 × 6–9 µm, broadly cylindrical, 8-spored, septate at base. Spore 15–18 × 5.5–8.5 µm, elliptic-fusiform.

***Hypoxylon rubiginosum* (Pers.) Fr:** Basidiocarp annual to perennial, resupinate, 0.9–16.2 × 0.5–6.9 × 0.1–0.3 cm, hard when fresh, brittle when dry purplish brown to amaranth. Fertile surface papillate, rusty brown when fresh, purpulish black when mature. Context homogenus. Peritheca 200–400 × 1500–4000 µm, spherical to obovoid, black. Ostioles are lower than stromatal surface umbilicate, inconspicuous. Asci cylindrical, 8-spored, septate at base, hyaline. Spore 11–15 × 5–6 µm, ellipsoid- inequilateral.

***Lentinus connatus* Berk:** Basidiocarp annual. Pileus 6.3 cm in diameter, plano-convex with slightly uplifted margin, smooth, faint creamy to yellowish orange when young, golden yellow to greyish brown when mature. Gills decurrent, 9–11 per cm, close to rather crowded, pink white to ochraceous. Stalk 5.6 × 0.7 cm, central, equal, solid, brittle, yellowish white to golden yellow. Hyphal system dimitic, generative hyphae 5.5–8 µm wide, skeleto-binding hyphae 2.5–5 µm wide. Spores 6–8 × 2.5–3 µm.

***Lentinus squarrosulus* Mont:** Basidiocarp annual, caespitose, gregarious. Pileus up to 6.2 cm in diam., infundibuliform, chalky white to pinkish buff to ochraceous.

squamose to squarrose. Gills 9–12 per cm, deeply decurrent, white to pale buff. Stalk 2.4–5.5 × 0.9–1.3 cm, central, eccentric also covered with flocculose sqamules, concolorous with pileus. Context up to 0.2 cm thick. Hyphal system dimitic, generative hyphae 2.5–4 µm wide, Skeleto-binding hyphae 2–2.5 µm wide. Spores 5–7.5 × 1.7–2.5 µm, cylindrical.

***Leucocoprinus birnbaumii* (Corda) Singer:** Basidiocarp annual. Pileus 2.9–4.6 cm in diameter, sub-globose, finally campanulate or expanded, truncate at apex, creamy white sometimes pale brown at centre. Gills free 11–14 per cm, moderately crowded, creamy white. Stalk 4.2–5.6×0.3–0.9 cm, cylindrical r swollen base, hallow, white powdery coating on the surface. Context thin, soft, chalky white. Annulus present. Hyphal system monomitic, generative hyphae 4.4–11 µm wide. Spores 7–10 × 5.5–6.5 µm, ellipsoid.

***Leucocoprinus cepistipes* (Sowerby) Pat:** Basidiocarp annual. Pileus 3.7–8.4 cm in diameter, obovoid then conical, finally comanulate or expanded, truncate at centre, chalky white with pale pink tints. Gills free 15–18 per cm, rather crowded, creamy white. Context thin, soft. Stalk 4.5–8.6 × 0.5–1.1 cm, cylindrical but broader below to give a sub-bulbous base, with powdery coating on the surface. Annulus present. Hyphal system monomitic, generative hyphae 3.5–5 µm wide. Spores 7–10 × 5–7 µm, ovoid.

***Leucocoprinus cretaceus* (Bull.) Locq:** Basidiocarp annual. Pileus 4.7 cm in diameter, conico-comanulate to umbonate or expanded, truncate at centre, chalky white with pale tints. Gills free 13–17 per cm, rather crowded, creamy white. Context thin, soft. Stalk 6.2 × 1.1 cm, cylindrical but broader below to give a sub-bulbous base, with powdery coating on the surface. Annulus present. Hyphal system monomitic, generative hyphae 3–5 µm wide. Spores 7–9.5 × 4.5–6.5 µm, ellipsoid to ovoid.

***Lopharia cinerascens* (Schwein.) G. Cunn:** Basidiocarp annual, crust-like, resupinate, effused-reflexed to pileate, 0.5–5.9 × 0.4–3.4 cm up to 0.1 cm thick. Pileus 0.5–4.1 × 0.3–0.5 cm up to 0.1 cm thick, sometimes semicircular, zonate, sulcate, velvety to tomentose, clay to smoky brown. Lower fertile surface, smooth, cracked when mature, olivaceous brown to smoky to brownish black. Context thin, homogenous. Hyphal system monomitic, generative hyphae 3.5–6 µm wide. Spores 7–9 × 3–4 µm, cylindrical to ellipsoid.

***Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden:** Basidiocarp annual, resupinate, 5.6–15.3 × 4.2–9.4 cm, up to 0.3 cm thick, membranous, creamy white to pale yellow brown. Fertile surface smooth, cracked on drying, creamy white to straw yellow when fresh, on drying pale yellow to pale yellow brown. Context papery thin.Hyphal system monomitic, generative hyphae 3.5–6.5 µm wide. Spores 5.5–8 × 3–5 µm, broadly ellipsoid.

***Phellinus allardii* (Bres.) S. Ahmad:** Basidiocarp perennial, resupinate to pileate, 9.8 × 7.9 × 4.3 cm. Pileus 1.4 × 0.5 cm, usually imbricate, reddish brown and covered with a tomentum. Lower fertile surface poroid, round, regular, pores 6–8 per mm, yellowish brown when young. Context very thin, sometimes almost absent. Tubes stratose, up to 0.2–0.4 cm deep in each layer, umber brown. Hyphal system dimitic, generative hyphae 2–3 µm wide, skeletal hyphae 3–4 µm wide. Spores 5–6 × 3.5–4 µm, broadly ellipsoid to subglobose.

***Phellinus badius* (Berk. ex Cooke) G. Cunn:** Basidiocarp perennial, pileate, sessile. Pileus 10.2 × 5.4 × 5.8 cm, hoof-shaped to ungulate. Upper sterile surface glabrous, weakly zonate, sulcate, yellowish brown to brownish black. Lower fertile surface poroid, round, pores 4–5 per mm. Context up to 2.1 cm thick, homogenous. Tubes up to 0.3 cm deep in each layer ferruginous brown. Hyphal system dimitic, generative hyphae 3–4 µm wide, skeletal hyphae 4–5 simple septate, wide. Spores 6.5–7.5 × 6–6.5 µm, broadly ellipsoid to subglobose.

***Phlebiopsis crassa* (Lév.) Floudas & Hibbett:** Basidiocarp annual, resupinate, 0.5–10.8 × 0.5–6.7 × 0.1–0.2 cm thick when fresh, purplish pink to purplish grey to pale violet to violet brown to brownish grey. Fertile surface when young velvety gradually surface become smooth, cracked on drying, grayish violet to purplish grey to violet to grayish brown. Context papery thin on drying, dense, smooth, homogenous. Hyphal system monomitic, generative hyphae 2.5–8.5 µm wide. Spores 6–8 × 3–4 µm, narrowly ellipsoid.

***Phylloporia pectinata* (Klotzsch) Ryvarden:** Basidiocarp perennial, pileate, solid. Pileus 14.2 × 9.1 × 0.7–5.8 cm, semicircular, applanate, sulcate, brownish yellow to brownish black. Lower fertile surface poroid, round, pores 5–6 per mm, yellowish brown to dark brown. Context up to 1.1 cm thick, duplex, yellowish brown to almost blackish. Tubes up to 0.3 cm deep, arranged in layer yellowish brown to brown. Hyphal system monomitic, generative hyphae 1.5–5 µm wide. Spores 3–3.5 × 2–3 µm, globose to sub-globose.

***Pleurotus ostreatus* (Jacq.) P. Kumm:** Basidiocarp annual, pileate. Pileus 7.1 × 4.8 cm, pleurotoid, spathulate to flabelliform, grey white to cream white. Gills decurrent, 7–10 per cm, white to grey white. Stalk reduced or sub-stipitate 0.7 × 0.6 cm, cylindrical, lateral or ecentric, creamy white at base, slightly greyish at upper part. Context thin, homogenous creamy white. Hyphal system monomitic, generative hyphae 3–6.5 µm. Spores 7.5–12 × 2–4 µm, cylindrical.

***Pseudofavolus tenuis* (Fr.) G. Cunn:** Basidiocarp annual, resupinate, effused-reflexed to pileate, 2.2–5.3 × 0.8–3.6 × 0.1–0.3 cm. Pileus 2.1–5.2 × 1.4–3.1 × 0.1–0.3 cm, semicircular, glabrous, zonate, sulcate, dark brown. Lower fertile surface poroid 1–2 per mm wide, hexagonal, brownish grey. Context up to 0.1 cm wide. Tubes up to 0.2 cm long. Hyphal system trimitic, generative hyphae up to 3 µm wide, skeletal hyphae 2–4.4 µm wide, binding hyphae up to 3µm wide. Spores 8.8–14.7 × 2.9–4.4 µm, cylindrical.

***Pycnoporus sanguineus* (L.) Murrill:** Basidiocarp annual, effused reflex to pileate. Pileus 2.3 –6.2 × 1.2–3.7 × 1.1–2.2 cm thick at the base, semicircular, glabrous, smooth, sulcate, pale orange to cinnabar red. Lower fertile surface poroid, 4–6 per mm pores, round, tomato red to brick red. Context up to 1.9 cm thick near base. Tubes up to 0.3 cm wide. Hyphal system trimitic, generative hyphae hyaline, 2.5–3.5 µm wide, skeletal hyphae 2–5 µm wide, binding hyphae 2–3 µm wide. Spores 5–6 × 2–2.5 µm, cylindrical.

***Scytinostroma duriusculum* (Berk. & Broome) Donk:** Basidiocarp annual, resupinate, 0.5–12.2 × 0.5–6.7 cm, up to 0.5 cm thick when fresh, creamy white to ochraceous. Fertile surface smooth, when touched gives hair-like or velvety sensation, creamy white to dull yellow when fresh, on drying pale yellow to ochraceous. Context thin, smooth, homogenous, pale yellow to dull yellow. Hyphal system dimitic, generative hyphae 1–4 µm wide, skeletal hyphae 1–6.5 µm wide. Spores 5–7 × 4.5–7 µm, globose to subglobose.

Schizophyllum commune **Fr:** Basidiocarp annual, pileate. Pileus 0.5–3.3 × 0.5–3 cm, 0.2–0.4 cm thick near the base, flabelliform to kidney shape, semicircular, velvety with tufts of woolly small hairs, greyish to dark greyish brown. Lower fertile surface falsely gilled, separating along the gill's edge, dichotomously branched, and greyish brown. Context up to 0.1 cm wide. Pseudogills 0.3 cm thick. Hyphal system monomitic, generative hyphae 4–7.5 µm wide. Spores 4–7 × 2–3 µm, suballantoid.

***Trametes cingulata* Berk:** Basidiocarp annual, pileate. Pileus 2.8–5.3 × 1.4–3.9 × 0.9–1.4 cm, semicircular to applanate, glabrous, zonate, sulcate, yellow white, later becoming sooty black. Lower fertile surface poroid, 3–6 per mm pores, round, shiny on the light incident, yellowish white to pale yellow. Context up to 0.7 cm thick. Tubes up to 0.5 cm wide. Hyphal system trimitic, generative hyphae 2–3µm wide, skeletal hyphae 3–5µm wide, binding hyphae 1–3µm wide. Spores 4–5 × 3–3.5µm, broadly ellipsoid.

***Trametes ellipsospora* Ryvarden:** Basidiocarp annual, resupinate to effused reflex to pileate. Pileus 2.4–5.2 × 1.6–3.9 × 0.1–0.4 cm thick, semicircular, applanate, strigose hairs, shiny, sulcate, weakly zonate, yellow white. Lower fertile surface poroid 3–5 per mm pores, angular, iripicoid, cream to ochre orange. Context up to 0.2 cm thick. Tubes up to 0.2 cm wide. Hyphal system trimitic, generative hyphae 2–3 µm wide, skeletal hyphae 2.5–5.5 µm wide, binding hyphae 1.5–3.5 µm wide. Spores 3–5 × 2–3.5 µm, ellipsoid.

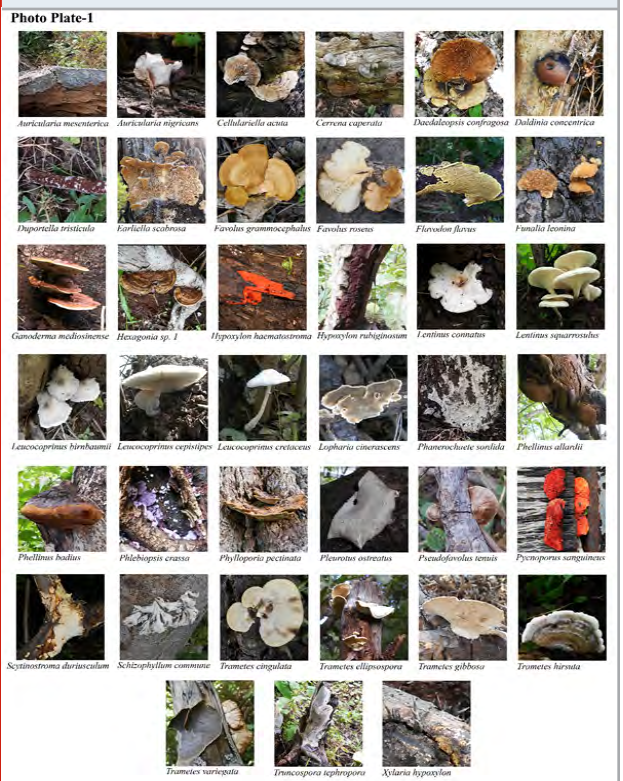
***Trametes gibbosa* (Pers.) Fr:** Basidiocarp annual, pileate. Pileus 8.4–12.3 × 4.1–6.8 × 0.5–1.4 cm, semicircular, applanate, glabrous, light zonate, sulcate, cream white to ochraceous. Lower fertile surface poroid 1–4 per mm wide, lamellate to deadaleoid or maize like, cream white to pale straw. Context up to 1 cm wide. Tubes up to 0.4 cm long. Hyphal system trimitic, generative hyphae 2–4 µm wide, skeletal hyphae 3–5 µm wide, binding hyphae 2–4 µm wide. Spores 4–5.5 × 2–2.4 µm, oblong ellipsoid.

***Trametes hirsuta* (Wulfen) Lloyd:** Basidiocarp annual, pileate. Pileus 4.2 × 2.3 × 0.1–0.4 cm thick, semicircular, applanate, hirsute, sulcate, weakly zonate, greyish white to greyish faint brown. Lower fertile surface poroid 3–4 per mm pores, circular to angular, cream to greyish white. Context up to 0.2 cm thick. Tubes up to 0.2 cm wide. Hyphal system trimitic, generative hyphae 2.5–4.5 µm wide, skeletal hyphae 3–5.5 µm wide, binding hyphae 2–3.5 µm wide. Spores 6–8.5 × 2–3 µm, cylindrical.

***Trametes variegata* (Berk.) Zmitr:** Basidiocarp annual, effused-reflexed to pileate. Pileus 2.8–3.7 × 2.8–3.1 × 0.1–0.2 cm, papery thin, weakly sulcate, zonate, papery thin, greyish brown to voilet brown. Lower fertile surface poroid 1–2 per mm wide, hexagonal to angular, brownish grey to cream grey. Context up to 0.1 cm wide. Tubes up to 0.1 cm long. Hyphal system trimitic, generative hyphae hyaline, 2–4 µm wide, skeletal hyphae 1.5–3 µm wide, binding hyphae 1.5–3 µm wide. Spores 9–13 × 4–4.5 µm, cylindrical.

***Truncospora tephropora* (Mont.) Zmitr:** Basidiocarp perennial, resupinate, 5.6 – 47.2 × 2.9 – 10.3 cm up to 1.9 cm thick at centre, hard when fresh, woody hard on drying. Lower fertile surface poroid, 4–6 per mm pores, round, greyish yellow to blonde. Context papery thin to almost absent. Tubes 0.1–1.9 cm wide, duplex or in a layer, light brown to coffee brown. Hyphal system trimitic, generative hyphae 2–3.5 µm wide, skeletal hyphae 3–4.5 µm wide, binding hyphae 1.5–3 µm wide. Spores 4.5–6 × 3–4.5 µm, broadly ellipsoid.

Figure 1: Photo plate -1



***Xylaria hypoxylon* (L.) Grev:** Basidiocarp annual, erect, up to 2.3 cm in length, corky, flattened, simple or branched, the lower part grayish black to black. Perithecia develop beneath the sporocarp surface showing protruduing papillae of the perithecial necks. Perithecia with comspicuous ostioles. Asci cylindrical, 95–115 × 5–5.5 µm, 8-spored. Ascospores 11– 14.5 × 5–6 µm, ellipsoid-inequilateral.

Table 1. Distribution and host name of wood-decaying fungi from Ajanta forest

Sr.no	Scientific name	Host	Date	Altitude	Latitude & Longitude	Collection Number
1	*Auricularia mesenterica* (Dicks.) Pers.	*Boswellia serrata* Roxb.ex Colebr.	15/11/19	502m	20°32'55"N 75°42'05"E	VUG/VPM-801
2.	*Auricularia nigricans* (Sw.) Birkebak, Looney & Sánchez-García.	*Azadirachta indica* A.Juss.	04/09/14	554m	20°32'53"N 75°42'13"E	VUG/VPM-84
3.	*Cellulariella acuta* (Berk.) Zmitr. & Malysheva,	*Tectona grandis* L.f.	20/08/16	551m	20°32'55"N 75°42'10"E	VUG/VPM-257
4.	*Cerrena caperata* (Berk.) Zmitr.	*Pistacia integerrima* J. L. Stewart ex Brandis	04/09/16	551m	20°32'55"N 75°42'10"E	VUG/VPM-345
5.	*Daedaleopsis confragosa* (Bolt : Fr.) Schroet	*Butea monosperma* (Lam.) Taub.	18/07/21	508m	20°32′57″N 75°42′05″E	VUG/VPM-815
6.	*Daldinia concentrica* (Bolton) Ces. & De Not.	*Pistacia integerrima* J. L. Stewart ex Brandis	02/10/19	418m	20°33′01″N 75°42′09″E	VUG/VPM-726
7.	*Duportella tristicula* (Berk. & Broome) Reinking	*Pistacia integerrima* J. L. Stewart ex Brandis	30/10/16	557m	20°32′53″N 75°42′13″E	VUG/VPM-686
8.	*Earliella scabrosa* (Pers.) Gilb. & Ryvarden	*Boswellia serrata* Roxb.exColebr.	15/11/19	500m	20°32'55"N 75°42'07"E	VUG/VPM-799
9.	*Favolus grammocephalus* (Berk.) Imazeki	*Bougainvillea spectabilis* Willd	18/07/21	419m	20°36′06″N 75°42′04″E	VUG/VPM-820
10.	*Favolus roseus* Lloyd,	*Nyctanthes arbor-tristis* L.	18/07/21	419m	20°36′06″N 75°42′04″E	VUG/VPM-819
11	*Flavodon flavus* (Klotzsch) Ryvarden	*Santalum album* L.	30/10/16	558m	20°32'55"N 75°42'10"E	VUG/VPM-689
12.	*Funalia leonina* (Klotzsch) Pat.	*Tectona grandis* L.f.	15/11/19	503m	20°32'56"N 75°42'06"E	VUG/VPM-796
13.	*Ganoderma mediosinense* J.D. Zhao	*Peltophorum pterocarpum* (DC.) K.Heyne	04/09/16	410m	20°33′09″N 75°42′03″E	VUG/VPM-349
14.	Hexagonia sp. 1	*Pistacia integerrima* J. L. Stewart ex Brandis	20/08/16	551m	20°32'55"N 75°42'10"E	VUG/VPM-255
15.	*Hypoxylon haematostroma* Mont	*Peltophorum pterocarpum* (DC.) K.Heyne	20/08/16	411m	20°33′04″N 75°42′03″E	VUG/VPM-260
16.	*Hypoxylon rubiginosum* (Pers.) Fr.	*Hardwickia binata* Roxb.	02/10/19	521m	20°32′54″N 75°42′10″E	VUG/VPM-727
17.	*Lentinus connatus* Berk	*Tectona grandis* L.f.	20/08/16	550m	20°32′55″N 75°42′11″E	VUG/VPM-259
18.	*Lentinus squarrosulus* Mont	*Nyctanthes arbor-tristis* L.	18/07/21	418m	20°33′04″N 75°42′06″E	VUG/VPM-904
19.	*Leucocoprinus birnbaumii* (Corda) Singer	*Senna siamea* (Lam.) H.S.Irwin & Barneby	18/07/21	501m	20°32′57″N 75°42′07″E	VUG/VPM-816

Table 1 Continue

20.	*Leucocoprinus cepistipes* (Sowerby) Pat	*Senna siamea* (Lam.) H.S.Irwin & Barneby	18/07/21	500m	20˚32′58″N 75˚42′06″E	VUG/VPM-818
21.	*Leucocoprinus cretaceus* (Bull.) Locq.	*Senna siamea* (Lam.) H.S.Irwin & Barneby	18/07/21	501m	20˚32′57″N 75˚42′07″E	VUG/VPM-811
22.	*Lopharia cinerascens* (Schwein.) G. Cunn	*Nyctanthes arbor -tristis* L.	30/10/16	557m	20˚32′53″N 75˚42′13″E	VUG/VPM-687
23.	*Phanerochaete sordida* (P. Karst.) J. Erikss. & Ryvarden	*Santalum album* L.	30/10/16	558m	20°32'55"N 75°42'10"E	VUG/VPM-690
24.	*Phellinus allardii* (Bres.) S. Ahmad	*Butea monosperma* (Lam.) Taub.	20/08/16	556m	20˚32′53″N 75˚42′14″E	VUG/VPM-254
25.	*Phellinus badius* (Berk. ex Cooke) G. Cunn	*Peltophorum pterocarpum* (DC.) K.Heyne	20/08/16	432m	20˚33′09″N 75˚42′02″E	VUG/VPM-264
26.	*Phlebiopsis crassa* (Lév.) Floudas & Hibbett	*Tectona grandis* L.f.	20/08/16	550m	20˚32′55″N 75˚42′11″E	VUG/VPM-258
27.	*Phylloporia pectinata* (Klotzsch) Ryvarden	*Senna siamea* (Lam.) H.S.Irwin & Barneby	02/10/19	442m	20˚36′06″N 75˚42′04″E	VUG/VPM-725
28.	*Pleurotus ostreatus* (Jacq.) P. Kumm.	*Pistacia integerrima* J. L. Stewart ex Brandi	30/10/16	410m	20˚33′11″N 75˚42′05″E	VUG/VPM-696
29.	*Pseudofavolus tenuis* (Fr.) G. Cunn	*Pistacia integerrima* J. L. Stewart ex Brandis	30/10/16	557m	20˚32′53″N 75˚42′13″E	VUG/VPM-685
30.	*Pycnoporus sanguineus* (L.) Murrill	On Angiospermic wood	25/10/21	410m	20˚33′02″N 75˚42′11″E	VUG/VPM-908
31.	*Scytinostroma duriusculum* (Berk. & Broome) Donk	*Bougainvillea spectabilis* Willd.	20/08/16	410m	20˚33′07″N 75˚42′05″E	VUG/VPM-266
32.	*Schizophyllum commune* Fr	*Peltophorum pterocarpum* (DC.) K.Heyne	20/08/16	501m	20˚32′58″N 75˚42′05″E	VUG/VPM-267
33.	*Trametes cingulata* Berk	*Pistacia integerrima* J. L. Stewart ex Brandis	01/10/21	501m	20˚32′55″N 75˚42′06″E	VUG/VPM-855
34.	*Trametes ellipsospora* Ryvarden	*Hardwickia binata* Roxb.	02/10/19	439m	20˚32′54″N 75˚42′10″E	VUG/VPM-728
35.	*Trametes gibbosa* (Pers.) Fr.	*Pistacia integerrima* J. L. Stewart ex Brandis	25/12/21	500m	20˚32′57″N 75˚42′07″E	VUG/VPM-862
36.	*Trametes hirsuta* (Wulfen) Lloyd.	*Pistacia integerrima* J. L. Stewart ex Brandis	06/09/21	410m	20˚33′08″N 75˚42′05″E	VUG/VPM-844
37.	*Trametes variegata* (Berk.) Zmitr	*Peltophorum pterocarpum* (DC.) K.Heyne	30/10/16	453m	20˚33′07″N 75˚42′03″E	VUG/VPM-693
38.	*Truncospora tephropora* (Mont.) Zmitr	*Tectona grandis* L.f.	01/10/21	503m	20˚32′58″N 75˚42′07″E	VUG/VPM-854
39.	*Xylaria hypoxylon* (L.) Grev	*Pistacia integerrima* J. L. Stewart ex Brandis	06/09/21	410m	20˚33′08″N 75˚42′05″E	VUG/VPM-845

CONCLUSION

In this study we are reporting for the first time the diversity of wood-decaying fungi from Ajanta Forest, which consists of 28 genera and 39 species. Among these 35 species were annual and 4 species were perennial. Most dominating to rarely observed genus were *Trametes* Fr (5 species) followed by *Leucocoprinus* Pat (3 species), *Auricularia* Bull (2 species), *Favolus* Fr (2 species), *Hypoxylon* Bull (2 species), *Lentinus* Fr (2 species), *Phellinus* Quél (2 species), whereas *Cellulariella* Zmitr. & Malysheva, *Cerrena* S.F. Gray, *Daedaleopsis* Schroet, *Daldinia* Ces. & de Not, *Duportella* Pat, *Earliella* Murrill, *Flavodon* Ryvarden, *Funalia* Pat *Ganoderma* P. Karst, *Hexagonia* Pollini, *Lopharia* Kalchbr. & MacOwan, *Phanerochaete* P. Karst, *Phlebiopsis* Julich, *Phylloporia* Murrill, *Pleurotus* (Fr.) P. Kumm, *Pseudofavolus* Pat, *Pycnoporus* P. Karst, *Scytinostroma* Donk, *Schizophyllum* Rr, *Truncospora* Pilát, and *Xylaria* Hill ex Schrank represent single genera.

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Relationship Between Body Mass Index, Cardiovascular Fitness and Physical Activity Among Computer Professionals of Three India Cities

Aman Goswami[1], Alpna Ahuja[2] and Mohammad Miraj[3]

[1]Consultant Physiotherapist, Holy Basil Hospital, Mohali, Chandigarh, Punjab

[2]Assistant Professsor, Saket College of Physiotherapy, Panchkula, Haryana

[3]Assistant Professor, Department of Physical Therapy and Health Rehabilitation, College of Applied Medical Sciences, AlMajmaah, Saudi Arabia

ABSTRACT

Cardiorespiratory fitness and physical activity are crucial health indicators that contribute to an individual's optimal physical performance. Research evidences show that relationship between body mass index, cardiovascular fitness, and physical activity has not been much researched and therefore the objective of this study was to examine the correlation between physical fitness, cardiovascular fitness, and body mass index among computer professionals. The cross-sectional study was done on 152 computer software professionals drawn from three Indian cities (Chandigarh, Panchkula and Mohali). Inclusion criteria included computer professionals between 21- 45 years working on computer for more than 8 hours per day having work experience of more than 1 year and working in company for more than 6 months. Different parameters for physical examination including blood pressure, weight and height measurements and body mass index for each subject was evaluated. Similarly, Physical activity level (in MET) was calculated using International physical activity questionnaire (IPAQ) and likewise cardiovascular fitness was evaluated by performing YMCA step test. The participants were classified into three BMI groups and association between body mass index, cardiorespiratory fitness and physical activity was determined using Pearson correlations at a significance level of $p \leq 0.05$. The study showed a significant negative correlation is seen between the body mass index and YMCA score among computer professionals suggesting that as compared to their normal-weight counterparts, overweight and obese computer professionals exhibited diminished muscular strength and physical activity along with cardiorespiratory fitness. Thus, It is imperative to develop interventions that specifically target these critical elements of physical fitness among such professionals.

KEY WORDS: RELATIONSHIP; INDEX; CARDIOVASCULAR; FITNESS; PHYSICAL COMPUTER; PROFESSIONALS.

INTRODUCTION

India is one of the leading country in the world with its IT industry been a major service sector since last two decades (Pandey et al.,2020; Shobha, Shibi and Shireen, 2016) According to NAASCOM, India's tech industry is estimated to touch $245 billion in the financial year 2023 and has been one of the major recruiter for the computer software professionals(Economic Times Report,2023). However, large working hours, sedentary and poor lifestyle among the computer professionals has also ushered a new set of occupational health problem where erractic working hours, deadlines compliance associated with prolong computer usage have been correlated with overweight and obesity(Genin et al.,2018).

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Globally the prevalence rate of overweight and obesity has been increased two fold since 1980 to the point where now one third of world's population can be categorized as obese. The prevalence of obesity in India is increasing with urban sectors having more prevalence than rural and females been more affected than their male counterparts (Saha et al, 2023). According to a systematic review of obesity in India, greater than 135 million people are affected with obesity. In comparison to rural areas, urban areas with high socioeconomic status are found to have higher prevalence(Verma et al.,2021; Ahirwar and Mondal.,2019). On determining the type of obesity, it was found that the prevalence of abdominal obesity is higher in India than the generalized obesity and again, urban areas have much higher prevalence than rural areas (Gupta et al., 2023).

Research studies also show that technological advancements have reduced physical activity, physical strain and energy expenditure, resulting in jobs that are more sedentary. As

many individuals spend a great deal of their waking hours in the workplace, so reducing sitting at work may need to be given special attention (Shrestha et al.,2016).Cardiovascular fitness of citizens of a country is a vital prerequisite to a country's realization of its full potentials. Technological developments and modern day commodities have navigated most people into sedentary life style leading to chronic diseases like hypertension, heart disease, diabetes mellitus, metabolic syndrome, chronic low backache& obesity, (Shrestha et al., 2016;Paterson et al.,2020).

Another study reported that adults with high leisure time internet and computer use were more likely to be overweight or obese as compared to participants who did not use the internet or computer. Previous studies reported that there is a significant negative correlation between obesity and VO2 max which indicated striking effect of increasing body fat on cardio-respiratory fitness (Paterson et al.,2020; Loh et al.,2020). Various studies showed effect of BMI on work related musculoskeletal discomfort but the relationship of BMI with physical activity level and cardiorespiratory fitness has not been extensively investigated (Bonney et.,2018; Patkar et al.,2022). So this study was designed to examine the relationship of BMI with cardiovascular and physical fitness in computer professionals.

MATERIAL AND METHODS

The cross-sectional study design included 152 IT professionals from IT companies in three cities of India (Chandigarh, Panchkula and Mohali). The inclusion criteria comprised of Computer professionals of age group 20 - 45 years and working on computer for more than 8 hours per day. Males were included with work experience of more than 1 year and working in company for more than 6 months. The exclusion criteria included History of diagnosed case of acute or chronic respiratory disorder, paralysis, major surgery, neurophysiological disorder, or on any regular medicine. Apart from that Software Professionals who were smokers and had night shifts were also not included in the study.

Before the commencement of the study, a formal approval for the study was taken from the Research and Ethics Committee and willing subjects were asked to give informed consent for the study. The physical examination {blood pressure, weight and height measurements and body mass index of each subject was documented. (Physical activity level (in MET) was calculated using International physical activity questionnaire (IPAQ).Subjects performed YMCA step test according to described protocol (YMCA Testing Report, 2000).

The procedure to perform YMCA bench step test was as follows where Metrone was set to 96 beats per minute and subject was asked to face the step. Stopwatch was started as the subject start to step on the step following the metronome beat following a cadence of up, up, down, down. This was continued for three minutes. After three minutes subject was asked to stop and immediately, sit on the step or stool and perform the manual pulse reading and count the number of beats for an entire 60 seconds. Pulse rate was taken from the radial pulse and recorded for 60 seconds and compared with the YMCA 3 Minute Step Test scoring.

Statistical Analysis: Statistical software package called IBM SPSS statistics (version 22.0) was utilized for analysis of data. Values are reported as Mean ± Standard deviation. The findings of BMI, IPAQ and YMCA bench step test were correlated statistically. Spearman's correlation was used to see the correlation of different variables. Level of significance is 95% so $p \leq 0.05$ is considered a significant result and p value< 0.01 is considered highly significant result.

Table 1. Demographic profile of the participants

Characteristics	Mean ± S.D
Total number of subjects (n)	152
Age (year)	24.08 ± 2.113
Height (meter)	1.736 ± 0.061
BMI (kg/m^2)	23.162 ± 2.744
SBP (mm Hg)	126.769 ± 5.479
DBP (mm Hg)	86.961 ± 6.415

Table 2: Distribution of subjects on the basis of BMI (in percentage)

BMI categories	Percentage of subjects
Normal	84.61%
Overweight	13.46%
Obese	1.92%

Table 3: Evaluation of PHR (post exercise), YMCA score and IPAQ BMI= Body Mass Index, PHR= Peak Heart Rate, YMCA= Young Men's Christian Association, MET= Metabolic equivalent of task, IPAQ= International Physical Activity Questionnaire

Variables	Mean ± S.D
PHR (post exercise) (bpm)	113.67 ± 16.674
YMCA score	1.88 ± 1.078
IPAQ (MET.min/week)	1033.423 ± 890.003

Table 4. Distribution of subjects having low, moderate and high physical activity as calculated from IPAQ

Physical Activity	Percentage of subjects
Low	48.07%
Moderate	48.07%
High	3.84%

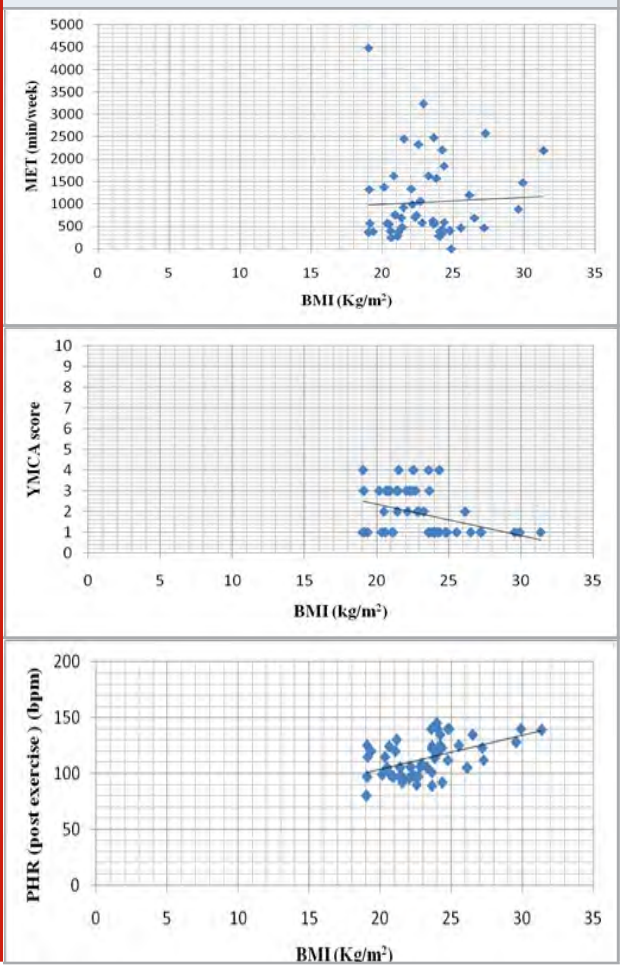
Table 5. Correlation of BMI with MET, YMCA and PHR

Variables	rs	p- value
BMI-MET	0.046	0.745
BMI-YMCA score	-0.417	0.002
BMI-PHR (post exercise)	0.482	0.000

RESULTS AND DISCUSSION

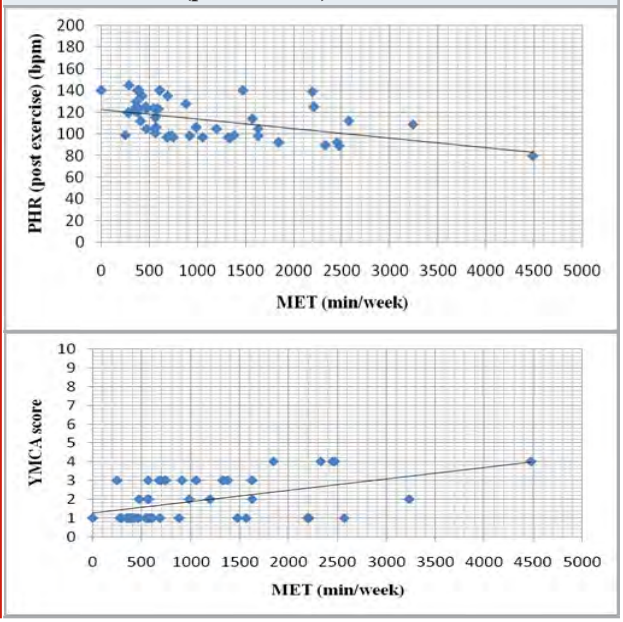
The demographic data of the 152 participants as well as distribution of the subjects on basis of BMI was described in Table 1 and 2 respectively. Similarly the evaluation of the participants based on Cardiovascular fitness and Physical activity was shown in Table 3 and 4 respectively. The correlation between different parameters i.e BMI-MET, BMI with YMCA score and PHR (post exercise) as well as MET with PHR (post exercise) and YMCA score was shown in Table 5. The scatter diagram for the different correlations were explained in Fig. 1.The Fig 1A showed the scatter diagram depicts a negative correlation between BMI and YMCA score.

Figure 1: showing Scatter diagram with Correlation between BMI with MET, YMCA and PHR (post exercise)



This scatter diagram 1B showed a positive correlation between BMI and PHR (post exercise) and likewise scatter diagram 1C showed a positive correlation between BMI and PHR (post exercise). Similarly, in Fig 2 different scatter diagram depicted correlation between MET and different variables such as PHR as well as YMCA score. The Fig 2 A showed a negative correlation between MET and PHR (post exercise) whereas Fig 2B showed scatter diagram having a positive correlation between MET and YMCA score.

Figure 2: showing Scatter diagram with Correlation between MET with PHR (post exercise) as well as YMCA score



Mean age of the subjects is found to be 24.08 and mean BMI of subjects studied is 23.162 (Table 1). Out of total 152 subjects who were measured, 84.61% were of normal BMI, 13.46% was overweight and 1.92 % was obese (Table 2, Fig. 1).Physical activity level of subjects was evaluated using International physical activity Questionnaire. Mean of METs calculated from IPAQ is found to be 1033.42 METs. min/week (Table 3). Out of total 152 subjects, 48.07% were having low level of physical activity, 48.07% had moderate level of physical activity and 3.84% had high level of physical activity according to IPAQ scoring protocol long form (Table 4,Fig. 2).

Cardiovascular fitness was evaluated using a YMCA bench step test and all the participants completed the test without any complications. Mean PHR (post exercise) and mean YMCA score after completing the test is found to be 113.67 and 1.88 respectively (Table 3) .On matching the scores of their performance with YMCA bench step test scoring12, it was found that the scores are between 1 and 2, which fall in the category of "Poor" or "Below Average". On correlating BMI with METs as calculated using IPAQ, there is weak positive correlation with BMI which was found to be statistically non-significant (Table 5 and Figure 2)

Based on the data from the computer professionals, the Body mass indexes of subjects included were correlated with peak exercise heart rate (post exercise), YMCA score

and Physical activity in METs.On matching the scores of their performance with YMCA bench step test scoring12, it was found that the scores are between 1 and 2, which fall in the category of "Poor" or "Below Average". Similarly, on correlating BMI with METs as calculated using IPAQ, there is weak positive correlation with BMI which was found to be statistically non-significant (Table 5, Fig. 2).

The findings of our study are in consistent with previous studies which showed no significant correlation between physical activity and obesity(Patkar and Joshi,2011). Another study also showed a weak negative correlation between IPAQ and BMI(Bonney et al.,2018). The reason behind this could be that the participants of this study were highly educated and aware about the health hazards of sedentary life style. Most of them were engaged in one or the other aerobic exercises. Moreover, the IPAQ questions reflect the physical activity level of past 7 days. However a different questionnaire reflecting the physically activity level of past one month would have modified the results. Furthermore, in yet another study conducted upon adults who engaged in high leisure-time internet and computer use were more likely to be overweight or obese even if they were highly active in their leisure time, as compared to participants who did not use the Internet or computer(Genin et al.,2018).

In the present study a significant negative correlation was observed between the body mass index and YMCA score of computer software professionals (Table 5, Fig 1). This indicates the striking effects of increasing body fat on cardiovascular fitness. This is supported by a previous study where the authors have reported that there is significant negative correlation between obesity and VO2 max(Shetty, Padmanabha and Doddamani, 2013). The reason behind this correlation could be that excessive amount of body fat exerts an unfavorable burden as well as hindering action toward cardiac function particularly during exhaustive exercise and excessive hyperactive body musculature fails to uptake sufficient amount of oxygen due to deposition of proportionately high amount of fat mass(Khona et al.,2017).

In the present study, a highly significant positive correlation was also observed between body mass index and peak heart rate (post exercise), (Table 5,Fig. 1).This was also in consistency with yet another study done where significantly positive correlation between body mass index and heart rate during Treadmill Jogging test was measured. It is known that obese people have increased sympathetic nerve firing rate than normal people. Thus, as heart is required to pump blood through relatively large depot of adipose tissue, Obesity leads to a state of chronic volume overload. Increased stroke volume and preload is related to hypertension and increased heart rate. Obese individuals with hypertension usually have thickening of ventricular wall and greater heart volume and so are more likely to suffer from cardiac failure (Dietrich et al.,2008). Obese subjects showed a significant increase in sympathetic activities of the heart, which showed imbalance in the autonomic neural activities of the heart(Bandyopadhyay and Chatterjee, 2003).

The current study found out significant negative correlation between MET and PHR (post exercise) (Table 6.Fig. 2).This showed that subjects who were physically active had decreased peak heart rate (post exercise).There are dearth of studies who correlated MET and PHR (post exercise). Aso study by Dietrich et al(2008) reported that middle-aged and elderly obese subjects who were regularly physically active had higher heart rate variability than their sedentary peers even after taking into account the effects of sex, age, study site, education, diabetes, hypertension, beta-blocker intake and smoking status. Reason behind is that regular physical exercise has strong beneficial effects on cardiac autonomic nervous function and thus appears to offset the negative effect of obesity on HRV. This study also found positive correlation between MET and YMCA score. This indicates the striking effects of increasing physical activity on improving cardiovascular fitness.

Regular exercise has a favorable effect on many of the established risk factors (sedentary life style, high blood pressure, abnormal values for blood lipids, smoking, and obesity) for cardiovascular disease. For example, exercise promotes weight reduction and can help reduce blood pressure. Exercise can reduce "bad" cholesterol levels in the blood (the low-density lipoprotein [LDL] level), as well as total cholesterol, and can raise the "good" cholesterol (the high-density lipoprotein level [HDL]).The Surgeon General's Report, a joint CDC/ ACSM consensus statement, and a National Institutes of Health report agreed that the benefits mentioned above will generally occur by engaging in at least 30 minutes of modest activity on most, preferably all, days of the week. Modest activity is defined as any activity that is similar in intensity to brisk walking at a rate of about 3 to 4 miles per hour.

These activities can include any other form of occupational or recreational activity that is dynamic in nature and of similar intensity, such as cycling, yard work, and swimming. This amount of exercise equates to approximately five to seven 30-minute sessions per week at an intensity equivalent to 3 to 6 METs (multiples of the resting metabolic rate*), or approximately 600 to 1200 calories expended per week21.Results of this study shows that with the increase of body mass index, post exercise peak heart rate increases suggesting low cardiovascular fitness of the population studied. Physical activity level is positively correlating with YMCA score suggesting that routine physical activity improves cardiovascular fitness in young population.

Limitations of the study and Future scope: The study had certain limitations too. For instance, the sample size was small, the female subjects were not included, Variables such as resting heart rate, heart rate recovery and blood pressure (post exercise) was not included, IPAQ records only activities done beyond 10 minutes, thus activities done less than that duration is not recorded thus it tends to underestimate the physical activity levels.The present study investigated the correlation with a small size so future research with a larger study population including both males and females is required for generalization of the results. The present study did not study variables like resting heart rate, heart rate recovery and blood pressure (post exercise), so

future research may be done to evaluate these variables also. Moreover, similar parameters of the study can be studied after a training protocol.

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Melanin Inhibitory Effects of *Aloe vera* Crude Extracts on the Isolated Scale Melanocytes of Zebrafish, *Danio rerio*

Gulafsha Kassab and Sharique A. Ali
Postgraduate Department of Biotechnology Saifia Science College, Bhopal, 462001, India

ABSTRACT

Increased production of melanin by stimulation of melanocytes leads to various hyperpigmented disorders, and the development of melanolytic agents causing hyperpigmentation is one of the highest priority areas of current research. Natural products with skin-whitening effects are gaining interest among consumers and researchers because they are perceived to be milder, safer, and healthier than synthetic alternatives. The present study was carried out to investigate the effects of crude extract of *Aloe vera* leaves, on the isolated scale melanocytes of zebrafish. In this study, the dorso-lateral scales of zebrafish were removed and kept in fish physiological saline with concentrations ranging from 0.02 to 1.52 µM. It was found that the crude extract of *Aloe vera* leaves induced powerful, dose-dependent, physiologically significant melanin aggregating effects in the isolated scale melanocytes of zebrafish as compared to control untreated embryos. At concentrations ranging from 0.02 to 1.52 µM, the MMSI of *Aloe vera* leaf extract was found to be 2.5 ± 0.14 to 0.5±0.14 as compared to the conrol value of 3.58±0.02. These preliminary outcomes clearly reveal that the crude extract of *Aloe vera* cause dose dependent melanin aggregation and result in skin lightening.

KEY WORDS: HYPERPIGMENTATION, MELANOCYTES, PIGMENTATION, ZEBRAFISH.

INTRODUCTION

Melanin is produced by melanocytes the pigment-producing cells, which serve a variety of vital physiological purposes, such as providing UV protection, preventing photocarcinogenesis, removing reactive oxygen species, and creating vitamin D. The accumulation of melanin-related skin issues such as melasma, post-inflammatory hyperpigmentation, skin cancer, and melanoma poses a major health concern (Agalou et al., 2018). To prevent aberrant melanin, whitening or lightening treatments are used. Dermatological research has been focusing on making new whitening agents from medicinal plants that do not have any adverse side effects in order to treat excessive and abnormal hyperpigmentation of the skin, and screening of plant-origin compounds is a top priority area of research.

Several chemically prepared compounds and molecules are used in cosmetic products for the inhibition of melanin (Kim et al., 2015; Ali and Parveen, 2021). Therefore, pigment cell researchers and dermatologists are now looking for a treatment that is safe, has no or very few side effects, and hardly contaminates the environment (Ali, 2017; Le et al., 2022). As the zebrafish genome has been fully sequenced and has 80% resemblance to the human genome, making it the most widely used model organism globally (Singh et al., 2019). Zebrafish also have melanin pigments on their bodies' surface, making it possible to see pigmentation without the need for a difficult experimental approach. As a result, the zebrafish is the perfect model to study pigmentation in the context of skin lightening (Jang et al., 2020). Hence, the use of herbs and their ingredients for the treatment of skin hyperpigmentation is gaining much interest as they are found to be safer, healthier, and milder than synthetic products.

Reviewing the literature, it becomes evident that extracts from the leaves of *A. vera* have been used traditionally as well as medicinally in various ailments such as wound healing, genital herpes, encephalitis, meningitis, eye infections, cold sore, diabetes, and AIDS (Ali et al., 2012; Mahor et al., 2016; Mahor et al., 2019). Many studies report protective effect of *Aloe vera* and some of its bioactive compounds especially aloin, also called barbaloin is a bitter tasting yellow crystal found in *Aloe vera*. It is the most important anthraquinone glycoside claimed to be responsible for beneficial effects of *Aloe vera* (Mahor and Ali 2018, 2019).

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The present study has provided vital information on these aspects, and very interesting results have been obtained. As there are no reports available in the literature on the effects of crude extracts of *Aloe vera* leaves, as skin lightening agents on the isolated scale melanocytes of zebrafish, this study aims to use zebrafish as an in-vitro animal model for the treatment of hyperpigmented disorders.

MATERIAL AND METHODS

The present research work was carried out in the laboratory of the Department of Biotechnology, Saifia Science College, Bhopal, India, and approved by the Institutional Animal Ethics Committee (IAEC) (approval number: SSC/IAEC/2022/02) under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, Government of India.

Collection and identification of plant material and prepare their extraction: *Aloe vera* leaves were obtained near Bhopal and authenticated from Botanical Society of India Kolkata, India, authentication number- CNH/Tech.II/2022/51. The *Aloe vera* leaves gel extraction was prepared according to the method of Sánchez-Machado et al., (2017) with slight modification. 250 gm of fresh *Aloe vera* gel was taken in a Soxhlet apparatus and the extraction process was done for 72 h at 40 °C temperature. The pooled extract was dry in a vacuum evaporator and the resulting dried extract stored at 4 °C. the lyophilized.

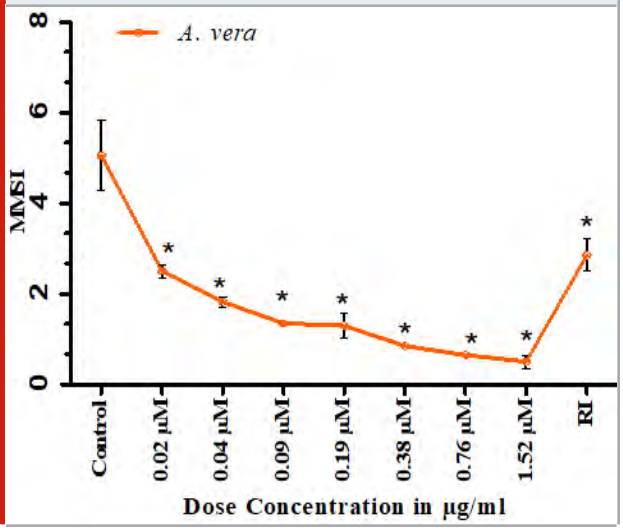
Zebrafish maintenance: Zebrafish were obtained from the commercial dealer in Bhopal, (M.P), India and kept in aquaria with provided proper aerating system to maintain the oxygen and water quality required for a healthy environment and then acclimatized for 15 days in an acclimatization tank. The temperature of the tank was maintained at 28±0.5°C, with light conditions of 14:10 hours. pH of tank water was maintained at 7.0±0.5. The zebrafish were fed twice daily with micropellet/ live food (size ±300 mm, Betta fish food and live brine shrimps). Sick and unhealthy fishes were removed from the acclimatization tank and transferred to the quarantine tank for proper care and treatment.

***In-vitro* fish scale preparation:** The zebra fish, *(Danio rerio)* scales were removed according to the method of Ali et al. (2011). Zebrafish were kept in a dry muslin cloth and selected dorsal lateral area of scales were removed with the help of a scalpel and kept in fish physiological saline containing [130 mM NaCl, 2.7 mM KCl, 5.6 mM D-glucose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM Tris-HCl, in 100 ml of double distilled water pH 7.2] in a small Petri dish and they were equilibrated in the saline medium for 15-20 minutes with frequent stirring. Dorsal lateral scales of zebrafish containing about 20-50 melanocytes were treated with various concentrations ranging from 0.02 to 1.52 µM crude extract of *Aloe vera*, untreated scales of zebrafish served as controls under same conditions.

The mean melanophore size index (MMSI) was assayed according to the method of Ali (1983) and Bhattacharya et al. (1976), which are modified versions of Hogben and Slome (1931) in which actual diameter (length×breadth) of ten randomly selected melanocytes of zebrafish scale was recorded using Leitz ocular micrometer, calibrated previously with 10×10 magnification. The value thus obtained was then multiplied by the unit of the micrometer, which was 15µ. Thereafter, the arithmetical mean was calculated. This was the mean melanophore size index (MMSI).

Statistical analysis: Statistical data analysis was presented as mean ± standard deviation n=7 represents the number of dose concentrations (treated) used for a particular experiment. Comparisons were made between the treated and control groups using student t-test. All data were analysed using Graphpad Prism 0.5 software $p<0.05$* indicates a statistically significant difference.

Figure 1: TDose-response curve for the melanocytes aggregating effect of crude extract of Aloe vera leaves. RI signifies the MMSI after the reimmersion of scale melanocytes of zebrafish in physiological saline after repeated washing. Vertical bars represent the standard error of mean p value <0.05.



RESULTS AND DISCUSSION

In the present study, depigmentation effect of crude extract of *Aloe vera* leaves was carried out on the isolated scale melanocytes of zebrafish.To further confirm the effect of crude extract of *Aloe vera* leaves on the isolated scale melanocytes of zebrafish, two parameters of melanogenesis were considered. First, microscopically observe the aggregation and dispersion pattern of scales melanocytes of zebrafish after treatment with different doses of crude extract of *Aloe vera* leaves. Second is to measure the mean melanophore size index (MMSI) in the treated isolated scale melanocytes of zebrafish *(Danio rerio)*.

In the present study the crude extract of *Aloe vera* leaves showed a comparable powerful melanin aggregating effect on the scale melanocytes of zebrafish with concentration ranging from 0.02 to 1.52 µM in which all melanocytes had

become perinuclear making the skin pale in colour when compared to untreated control. In response to the maximal concentration of 1.52 µM of the crude extract of *Aloe vera* leaves, the MMSI of the melanocytes found to be 0.5±0.14 as compared to the control value of 3.58±0.02 in which melanocytes were an intermediate state neither aggregated nor dispersed. After repeated washing and reimmersion (RI) of the *Aloe vera* extract treated melanocytes in fish physiological saline, it was found that the powerful melanin aggregation effects of the extract completely vanished, as the MMSI became 2.95±0.07 (Fig: 1). It was found that the crude extract of *Aloe vera* leaves is a powerful melanolytic agent in the scale melanocytes of zebrafish as compared to the control.

The distribution and synthesis of melanin by melanocytes in the epidermis determine skin pigmentation. However, an excess of melanin can cause a number of hyperpigmentary skin diseases that can seriously impair appearance (Mort et al., 2015). Numerous research studies have examined the application of natural products in cosmetics and medicine (Mahor and Ali, 2018). Thus, our aim was to identify new agents from natural sources that can regulate melanogenesis.

As there are no reported inhibitory effects of crude extract of *Aloe vera* on the skin melanocytes of zebrafish in literature, this study is the first one to elucidate the effect of melanin aggregation by crude extract of *Aloe vera*, induced skin lightning for creating new depigmentation substances for the treatment of hyperpigmentary disorders and to make the skin a shade lighter.

Our results indicated that, crude extract of *Aloe vera* leaves exerts significant skin lightening response in dose-dependent manner in the isolated scale melanocytes of zebrafish. It was found that crude extract of *Aloe vera* leaves aggregated all the isolated scale melanocytes of zebrafish significantly in a dose dependent manner (0.02 to 1.52 µM). At lower concentration, minimal effect of aggregation was perceived under microscope. On increasing concentration, more aggregation effect of melanocytes was observed. Isolated scale melanocytes treated with 0.76 µM of crude extract of *Aloe vera* showed high aggregation of melanocytes as compared to control. More dense aggregation was observed at 1.52 µM concentration of crude extract of *Aloe vera.*

At higher concentration melanocytes had become perinuclear making the skin pale in colour. Our findings are substantiated with the findings of Choi et al. (2002) who showed that aloesin from *Aloe vera* showed significant depigmentation property in human skin exposed to ultraviolet radiation. In our study, scales of zebrafish have been used to observe the aggregation pattern of melanocytes. This method is the preliminary effective method as used in our previous study (Ali and Naaz, 2014; Ali et al., 2012; Ali et al., 2023) where skin melanophores of *Bufo melanostictus* were used to study the skin pigmentation effect of berberine bioactive compound. The results indicated that the crude extract of *Aloe vera* leaves aggregated the melanocytes dispersion effect on the isolated scale melanocytes of zebrafish *(Danio rerio)*. In this study, we reported that *Aloe vera* induced powerful dose dependent physiologically significance melanin aggregation in the isolated scale melanocytes of zebrafish leading to lightening of the skin. The data of the present study clearly demonstrate that *A. vera* can have clinical application as nontoxic melanolytic agents for the treatment of hyper pigmentation.

CONCLUSION

It is concluded that the leaf extract of *Aloe vera* induced powerful, dose-dependent, physiologically significant melanin aggregating effects in the isolated scale melanocytes of zebrafish *(Danio rerio)*. This was confirmed by using control untreated embryos. It was observed that crude extract of *Aloe vera* leaves significantly aggregates melanin and make the zebrafish skin pale. Our study opens new vistas for the use of *Aloe vera* regarding its clinical application as a new nontoxic melanolytic agent for the treatment of hyperpigmentation.

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Name of the Scholar	Fellow Society for Science & Nature (FSSN) and Member BBRC	Designation and Address of the Scholar
Dr. Sharique A. Ali, FLS FRSB (UK)	FSSN / BBRC	Professor and Head, Department of Biotechnology Saifia Science College, 462001 Bhopal, India
Dr. Ayesha S. Ali	FSSN / BBRC	Professor Department of Biotechnology Saifia Science College, Bhopal 462001,India
Dr. J. Peter	FSSN / BBRC	Associate Professor, RKDF University Gandhi Nagar , Bhopal 462023 India
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Dr. Sarita Shrivastava	FSSN / BBRC	Professor of Chemistry, Govt Motilal Vigyan Mahavidyalya , Bhopal India
Dr. P.M. Makode	MSSN / BBRC	Associate Professor of Zoology, Venue, Park, Shegaon Naka VMV Road, Amravati (MS) India
Dr. Sunita Yadav	MSSN / BBRC	Assistant Professor Department of Zoology, Satya Sai College for Women BHEL, Bhopal India Bhopal
Dr. Romsha Singh	MSSN / BBRC	Associate Professor, Department of Zoology, MLB Girls College, Bhopal India
Dr Shaima Miraj PhD	FSSN / BBRC	College of Health Sciences, Saudi Electronic University Riyadh Saudi Arabia
Dr Sushma Prasad PhD	MSSN/BBRC	Zarifa Farm, Kachhwa Road, Karnal, Haryana 132001, India
Dr Kamal Zaidi PhD	MSSN/BBRC	Department of Microbiology Peoples University Peoples Campus, Bhanpur, Bhopal, 462037 India
Dr. A. D. Lakha	FSSN / BBRC	Associate Professor of Zoology, Nagazari Area, MIT Road, Ambajogai, Beed (MS) 431517 India
Dr. R. S. Virani	MSSN / BBRC	Associate Professor Karimabad Society, Pandhar Kawada, District, Yeobtmal (MS) India
Dr. Madhulika. Pal	MSSN / BBRC	Assistant Professor, Department of Biotechnology Sadhu Vaswani College Bhopal, India
Dr. V.R. Wankhede	MSSN / BBRC	Assistant Professor, Department of Zoology, Deccan College, Pune, India

Dr. Mrs. V. Ingole	MSSN / BBRC	Department of Zoology Vidya Bharti Mahavidyalaya, Amravati, India
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Dr. O.N. Tiwari	FSSN / BBRC	Senior Scientist, Department of Biotechnology NE Region Imphal, Manipur, India
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Dr Anjali Choudhary PhD	MSSN/BBRC	Department of Biochemistry Opposite to Dussehra Maidan, BHEL Square, Sector A, Govindpura, Bhopal, 462023 India
Dr. V. Meitei	FSSN / BBRC	Department of Biotechnology NE Region Imphal, Manipur, India
Dr. N. Qayyumi	MSSN / BBRC	Assistant Professor of Zoology, Mittal College Bhopal, India
Dr LK Jakkala PhD.	FSSN/BBRC	Director Macrocare, Macrocare Towers, Hyderabad Telangana 500081, India
Dr. M. Sajid	MSSN / BBRC	Head Department of Biotechnology Bonnifie College, Bhopal, India
Dr. V. Jaiswal	MSSN / BBRC	Research Scholar, SGB University, Amravati, India
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Dr. R. Khalique	MSSN / BBRC	Department of Zoology Holkar Science Colege, Indore India
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Dr. F Kabinwar	FSSN / BBRC	Professor of Oncology, University of California, Los Angeles, USA
Dr Neelu Qayyumi PhD	MSSN/BBRC	Professor and Head Life Sciences Mittal College Opposite Bhopal Memorial Hospital Bhopal India Hospital Research Centre (BMHRC), Navi Bagh, Karond, Bhopal, 462008 India
Dr. Arjun Deb	FSSN / BBRC	Professor of Zoology Lumding College Lumding Assam, India
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Dr. Sushil Kumar Upadhyay D. Phil	FSSN/BBRC	Assistant Professor, Department of Biotechnology Maharishi Markandeshwar (Deemed to be University) Mullana Ambala 133207 (Haryana) India

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