Biofortification of Zinc in *Pleurotus sp.*

BY

Shri Mathi K

Reg.No: 20PBO015

A Thesis Submitted to the

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore - 641-043.

In Partial Fulfillment of the Requirements for the Degree of

DEGREE OF MASTER OF SCIENCE IN BOTANY

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Signature of the
Head of the Department

Signature of Supervisor

Acknowledgement

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Introduction

Introduction

Agriculture is the main strength of the Indian economy. Various kinds of agricultural crops are grown, and food security is achieved by producing over 250 million tonnes of food grains. However, due to overgrowth in population, agricultural land reduction, environmental issues, water, manpower, etc., our struggle to achieve nutritional security is still on. There are many agrowastes from agricultural crops, approximately 600 million tonnes of agro wastes are produced in our nation per annum. These agro wastes are left out to decompose naturally or burnt in situ resulting in soil, water, and air pollution. One of the reusing agrowastes is the cultivation of mushrooms which uses agro wastes as substrates resulting in a protein-rich food substance with low calories, fat, and also cholesterol-free. The amount of fiber is also high, with lots of minerals in it. The spent mushroom, which is used up as substrate after mushroom cultivation, can be used as organic manure. This also helps to enhance income. India produces about 600 million tonnes of agro wastes per annum. A significant part of it is left to decompose naturally or burnt in situ, resulting in soil, water, and air pollution.

Mushroom belongs to kingdom Mycetae and is also mentioned as macro fungi. Early history shows mushrooms were consumed as medicine and longevity by Greek and Romans. Greeks believed that it provides strength to the warriors during the battle; Romans believed that mushrooms were “Foods of the Gods”; Chinese considered them an “elixir of life”, and Pharaohs considered them a delicacy. Later, mushrooms were considered to be rich man’s food and consumed by everyone because of their cultivation and commercialization.

In France, an effort to cultivate the macro fungus started in 1650 with the cultivation of *Agaricus bisporus* (Button mushroom) (Atkins, 1978; Patel, 2013), which spread to England, America, etc. Cultivation of various edible mushrooms for food and medicine happened for ages. *Pleurotus* sp. (Oyster mushroom) cultivation started on a Germany test premise by Flack during 1917 on tree stumps and wood logs (Ladli, 2020).

Among the different types of mushrooms cultivated, the White button mushroom stands first, next to the Shiitake mushroom, and third place goes to Oyster mushrooms (*Pleurotus*) (Gyorfi *et al*., 2007). Oyster mushrooms are widely cultivated in southeast Asia, India, Europe, and Africa. They can be cultivated and harvested throughout the year in both temperate and tropical regions. It is also mainly cultivated because of its easiest way of cultivation as it naturally colonizes fast. They grow on any lignocellulosic crop residues into protein-rich food with their biological

efficiency of 100%. *Pleurotus* mushrooms are in need because of their unique delicacy with high nutritional and medicinal values (Caglarirmark, 2007; Raman *et al*., 2021). Nowadays, mushroom consumption is increasing because of its high protein and dietary fiber composition (Singh and Singh, 2011; Bano and Srivastava, 1962) and essential and non-essential amino acids and minerals (Raman *et al*., 2021).

Mushroom cultivation has become an agribusiness profitable in many developing and developed countries. The cultivation of *Pleurotus* mushroom is going overwhelmingly in many ways, like substrate substitution or changing, to improve the quality of elements present in them. Enriching the substitute with minerals to enrich the constituents of minerals present in mushrooms is also carried on to improve the quality.

All living organisms are in need of macro and microelements. Their deficiencies result in various health issues (Blanco-Rojo and Vaquero, 2019). In order to overcome this problem nowadays, enrichment of soil in order to increase the content in plants and substrates with regard to mushrooms is done. Mushrooms are supplemented with essential elements to enrich the elemental components, which enhance other functional activities and also combat nutrient deficiency symptoms (Oyetayo *et al.,* 2021).

Zinc is a trace mineral that plays a vital role in many physiological activities. A deficiency is a worldwide problem causing a severe threat to human health (Faroog *et al*., 2018). The role of zinc in biology can be grouped into three general functional classes, namely catalytic, structural and regulatory functions (Cousins *et al*., 1996). Especially in developing countries, zinc is found to be less and creates lots of public health issues.

Mushrooms being macro fungi, have a greater mineral accumulating capacity. In order to combat the deficiency of zinc, mushrooms with zinc enrichment can be cultivated and consumed. The present study of the biofortification of zinc is done on three species of *Pleurotus (P. florida, P. eous* APK1 and *P. ulmarious* CO2*).* The objectives of the present study are as follows:

1. To enrich the three *Pleurotus* sp *(P. florida, P. eous* APK1 and *P. ulmarious* CO2*)* for Zinc using Zinc sulfate.
2. To compare the primordial development and other growth parameters of mushrooms with and without Zinc sulfate treatment.
3. To compare the effect of Zinc biofortification on the biochemical parameters.
4. To compare the effect of zinc biofortification on antioxidant activity.

Review of literature

Review of Literature

Fungi which belong to the kingdom *Myceteae*, are a large group of diverse organisms. Their existence is proved by fossil records and calculated to be in the Paleozoic era (408-438 million years ago) in the Silurian period (Alexopolus, Mims & Blackwell, 1996; Ren *et al*., 2012). Fungi are estimated to be of 2.2 - 3.8 million species. Among them, 144,000 are known to mankind (Hawksworth and Lucking, 2017; Cannon *et al*., 2018). Yeasts, smuts, rusts, mildews, and mushrooms belong to fungi. Fungus is beneficial, from which medicines, organic acids, and vitamins are synthesized, while few are pathogenic, causing various diseases in living organisms. Mushroom belonging to the saprophytic kind falls into both categories of fungi as few of them are edible while a few are poisonous. Most edible mushrooms belong to the phylum Basidiomycota and are also called macrofungi. The existence of mushrooms has a very long way back, proved by various literature like Veda and Bible. Theophrastus (372-287 BC), the great Greek philosopher, wrote about the value of mushrooms. Greek and Roman emperors, Considered mushrooms royal dishes during the middle ages.

Humans have consumed mushrooms from ancient times for various reasons. After a long time, they became a part of their diet because of their aroma, taste, high protein content, and medicinal properties (Zhong and Tang, 2004, Firenzuoli *et al*., 2008, De Silva *et al*., 2012a, 2012b and 2013). FAO has recommended edible mushrooms as food due to the protein content contribution in developing countries (Josephine, 2015). Mushrooms are high in vital bioactive phytonutrients and essential vitamins and minerals. They are also one of the greatest sources of sulphur inclusion in the diet, as they include the antioxidant amino acids ergothioneine and tripeptide glutathione (Dubost *et al*., 2006; Halliwell *et al*., 2018; Kalaras *et al*., 2017 and Pizzorno, 2014).

The macro fungi produce conspicuous sporocarps (Fruiting bodies). The large sporocarp of mushroom can be seen with the naked eye and picked up by hand (Chang, 2012). Worldwide, mushrooms are used as medicines and for daily consumption as food. From a culinary standpoint, mushrooms are considered vegetables and have been informally classified as "white vegetables" (Weaver and Marr, 2013). The USDA's MyPlate considers mushrooms to be part of the other vegetable's subgroup, and 1/2 cup of mushrooms counts as 1/2 cup equivalent in the vegetable group (other vegetable subgroup) (USDA, 2020). It has various health-promoting properties, and many experiments have proved to have antifungal activity, antigenotoxicity,

antihypertensive, antiproliferative, antioxidation, antihyperlipidemic activity, antitumorigenic, antiatherogenic, immunostimulant and also has stress-reducing properties.

Mushroom cultivation in the country started in the 70s, but with the development of the technologies for environmental controls and increased awareness about the nutritional factors, mushroom production has increased drastically. In 1990 the show was around 5000 tonnes 1990 (Singh and Kamal, 2021). The present statistics are given in table 1, given by the Indian state taken from the Ministry of Agriculture and Farmers Welfare.

Table: 1. Selected State-wise Production of Mushroom in India
(2020-2021-2nd Advance Estimartes)

States/UT	Production (In ' 000 Tonne)	States/UT	Production (In ' 000 Tonne)
Andhra Pradesh	3.00	Meghalaya	0.05
Arunachal Pradesh	0.08	Mizoram	0.07
Assam	1.40	Nagaland	0.50
Bihar	21.33	Odisha	22.50
Chhattisgarh	13.90	Punjab	18.50
Gujarat	14.50	Rajasthan	14.60
Haryana	19.60	Sikkim	0.02
Himachal Pradesh	14.80	Tamil Nadu	11.00
Jammu & Kashmir	3.50	Telangana	-
Jharkhand	7.50	Tripura	0.15
Karnataka	4.50	Uttar Pradesh	16.02
Kerala	1.00	Uttarakhand	14.00
Madhya Pradesh	1.50	West Bengal	7.00
Maharashtra	22.00	Others	9.75
Manipur	0.10	**India**	**242.85**

Source: Ministry of Agriculture and Farmers Welfare, Govt. of India. (ON2844)

Worldwide since ancient times, mushrooms have been considered and used as ‘the ultimate health food’ (King, 1993), and over 200 species of mushrooms are being used as a functional food (Kalac, 2013). Only 35 species are cultivated for commercial purposes (Aida *et*

al., 2009 and Xu *et al*., 2011). The nutritional value and sensory qualities of edible mushrooms are determined by their chemical composition (Shah *et al.*, (1997) Manzi *et al.*, (2001)). According to Breene (1990), Ço_kuner, and Özdemir (2000), mushrooms contain 19 to 39 g of protein in 100 g of dried mushrooms. Mushrooms are high in fiber and protein with lesser carbohydrates and saturated fats with minimal or no cholesterol. This combination of components helps in reducing harmful blood cholesterol, and it also acts as an appetite suppressant. They are also rich in minerals and vitamins B, C and D (Panjikkaran and Mathew, 2013).

Consumers value edible mushrooms not only for their flavour and aroma but also for their health-promoting properties. Mushrooms which are high in nonstarchy carbs and most amino acids, minerals, and vitamins, can be used in vegetarian diets as a meat substitute. Mushrooms may help overcome protein deficits in underdeveloped nations where high-quality proteins from animal sources are unavailable or inappropriate due to religious beliefs.

Mushroom cultivation is one of the most rapidly growing sectors of the modern food industry in many countries. Mushrooms are low in fat, and they are low-calorie foods and an essential source of nutrients and many bioactive compounds. Vitamin B, selenium, copper, potassium, and fibre are rich in mushrooms (Feeney, Dwyer *et al*., 2014). They are an abundant source of D vitamins when exposed to UV light (Kalaras *et al*., 2012). Varieties of phenolic antioxidants are present in mushrooms as secondary metabolites (Kalaras *et al*., 2017). For the past two decades, the world production of cultivated edible mushrooms has been around 7 million tonnes (Ijeoma *et al*., 2015). Cultivating mushrooms yields nutritious food and converts waste into high protein food (Thakur, 2020).

The most widely cultivated mushroom is *Agaricus*, second to its cultivation and commercial usage is *Pleurotus.* Pleurotus mushrooms, commonly called Oyster mushrooms, which belong to the family Pleurotaceae, are among the most popular edible mushrooms among all farmed edible mushrooms (Kong, 2004). *Pleurotus* species belongs to the class basidiomycetes, and it is grouped under “White rot fungi” (Tsujiyama and Ueno, 2013) since they produce a white mycelium, which is most extensively studied for its ligninolytic properties (Philippoussis *et al*., 2001; Olivieri *et al*., 2006 and Li and Shah, 2016).

There are various kinds of *Pleurotus* which are commercially cultivated having considerable economic value, which includes *P. ostreatus* (oyster mushroom), *P. eryngii* (king

oyster or Cardoncello), *P. pulmonarius* (Phenix mushroom), *P. djamor* (pink oyster mushroom), *P.sajor-caju* (Indian oyster), *P.cystidiosus* (abalone oyster), *P. citrinopieatus* (golden oyster mushroom) and *P. cornucopiae* (Pe´rez- Martı´nez *et al*., 2015; Knop *et al*., 2015 and Zhang *et al*., 2016). *Pleurotus* is a genus with approximately 40 species distributed over a wide range of temperate and tropical regions (Golak- Siwulska *et al.*, 2018). They are of different colours, including white, cream, grey, yellow, pink or light brown (Singh *et al.*, 2011). The scientific and common names both allude to the fruiting body's shape. The Latin word *Pleurotus* (sideways) refers to the stem's sideways development with the cap. Mushroom cultivation has increased (Royse, 2002 and Shelly *et al*., 2009).

Polypore mushrooms from the Basidiomycota and Pleurotus genera have a wide range of medical and cosmetic applications due to their immunostimulatory, anti-cancer, and anti-ageing characteristics. Because of its culinary worth and pharmacological effect, the King oyster mushroom, *Pleurotus eryngii,* is one of the best-known species in this genus (Vetvicka *et al*., 2019; Stamets *et al.,* 2011; Akyüz *et al.,* 2010*;* and Sekara *et al.,* 2015). It is related to the oyster mushroom (*Pleurotus ostreatus*), but the fruiting body morphology is distinct.

Table:2 Nutrient Profiles of Mushrooms (Oyster mushroom) Composites (per 84 g serving) (USDA/ARS, 2020b)

Energy (kcal)	27.7
Protein (g)	2.78
Carbohydrate (g)	5.12
Dietary Fiber (g)	1.93
Total Fat (g)	0.34
Saturated Fat (g)	0.05
Cholesterol (mg)	0.00
Calcium (mg)	2.52
Copper (mg)	0.20
Iron (mg)	1.12
Phosphorus (mg)	101

Potassium (mg)	353
Selenium (µg)	2.18
Sodium (mg)	15.1
Zinc (mg)	0.65
Vitamin A (µg)	1.68
Thiamine (mg)	0.105
Riboflavin (mg)	0.29
Niacin (mg)	4.16
Folate DFE (µg)	31.9
Vitamin B6 (mg)	0.09
Vitamin B12 (µg)	0
Vitamin C (mg)	0
Vitamin D (µg)	0.61
Vitamin E (mg)	0
Choline (mg)	40.9

In Asia, mushroom usage as food and medicine originates from centuries-old practices that have been more thoroughly documented than those in Europe. *Pleurotus* was first cultivated during the First World War in Germany as a subsistence measure for food storage, and the first documentation of cultivation was done by Kaufer (Kaufert *et al.* 1936). It is widely cultivated due to its medicinal and organoleptic properties, and the cultivation is simple with low-cost production and higher biological (Chirinang and Intarapichet *et al*., 2009). In comparison to other mushrooms, *Pleurotus* species require a short growth period. Pests and diseases rarely attack its fruiting body, and it can be grown quickly, sporeless, with high yield, broader substrate utilization, broader chemical and temperature tolerance, and environmental bioremediation.

Oyster Mushrooms (*Pleurotus spp*.) are among the most popular subtropical varieties. For temperate regions, *Pleurotus ostreatus, P. florida* (winter strain) and, *P. fossulatus* (Kabul dhingri), *P. eryngii* (King oyster) are ideal. The area suitable for button mushrooms is equally suitable for cultivating these species. Most oyster mushroom species are subtropical and grow well in the temperature range of 20-32°C. The most popular ones are *P. sajor-caju, P. florida, P.*

flabellate, and *P. eous*. Oyster mushroom is dried and can be exported in dried form. This variety is most suitable for production in India (Singh and Kamal, 2021).

Mushroom survival and multiplication have been linked to several elements that may work alone or in concert. Chemical composition, water activity, C:N ratio, pH, surfactant, minerals, moisture, nitrogen sources, particle size, inoculum amount, antimicrobial agents, and microorganism interactions are all considered chemical, physical, and biological factors that influence mushroom production (Eira, 2003). Temperature, humidity, brightness, and air composition of the surrounding substrate, such as oxygen and carbon dioxide concentrations, are the most critical environmental parameters (AMGA, 2004). The mushroom of the *Pleurotus* genus are delicate, and they are so sensitive and start deteriorating within a day after harvesting. Under normal refrigeration conditions, it can extend the mushroom's shelf life a few days. After harvesting, various physiological and morphological changes will occur, making the mushroom unavailable for consumption. Drying mushrooms is one of the crucial practices to extend their shelf life. Dried mushrooms are convenient for long term storage as well as for transportation. The dried form of mushroom is also helpful in various lab oriented mushroom processes. *Pleurotus* species require a short growth time compared to other edible mushrooms.

Pleurotus florida was introduced by Eger to Europe from the United States in 1973, and the ideal temperature for growing was 20-25°C. HOWEVER, when *P. florida* was imported to Japan, the cultivation temperature was set at 15-25°C. Then, in 1986, the Rural Development Administration (RDA) introduced it in South Korea under the local name SACHEOL NEOTARY. It was released for commercial cultivation in 1983 by the Department of Plant Pathology, TNAU, Coimbatore. Sporophores are white to light creamy white with good taste. Highly suitable for commercial cultivation under subtropical conditions. Preferred by growers due to uniform yield throughout the year. Consumers prefer soft creamy white mushrooms with good cooking quality. Bioefficiency: 120-130 per cent with cropping cycle of45 days.

Pleurotus ulmarius (Elm oyster mushroom) was introduced for commercial production for the first time in India by IIHR. Mushrooms are initially grey in colour, fading on maturity, Gills and stalks are white. Mushrooms are large and fleshy with excellent taste. Mushrooms occur in clusters. Its shelf life is 36-48 hours at 25-30°C and 4-5 days at 4°C. Commercial cultivation has been standardized on pasteurized (80-85°C for 2 hours)/sterilized (121 °C, 15 lb pressure for 15 minutes) paddy straw. It completes spawn run in 25-30 days in a temperature range of 25-30°C.

Pinhead initiation begins after 4-7 days of opening the bags and matures for harvest within 2-3 days. The total cropping cycle of this variety from spawning to harvesting is 37-42 days, which can obtain an average of 60-80% of its biological efficiency. It can be marketed as fresh, dry or as mushroom powder. For natural growing, any region in India has a temperature range between 20-25°C. Under a controlled environment, all the regions. All year round natural cultivation can be undertaken in Kodagu, Chikmagalur, Kodaikanal, Ooty, Coonoor, and Northeastern Manipur, Meghalaya Mizoram, Nagaland and Arunachal Pradesh. Seasonal cultivation can be done in other regions. Spent mushroom substrate (SMS) can be used as excellent organic manure or vermicomposting.

Generally, mushrooms contain more bioelements than vegetables and fruits, and they also show an adequate uptake of bioelements from the substrates. Substrates used in mushroom cultivation affect mushrooms chemical, functional and sensorial characteristics of mushrooms (Oyetayo and Ariyo, 2013). *Pleurotus spp*. is a saprophyte that extracts its nutrients from the substrate through the mycelium, obtaining substances necessary for its development, such as carbon, nitrogen, vitamins and minerals (Urben, 2004). Substrate plays an important role in protein content in mushrooms, variation in protein content is due to variation in the growing substrate of the mushroom. Leaching out during steeping and the browning reaction is also a reason for lower protein content in dried mushrooms. Usually, drying mushrooms may decrease their protein content in them (Hassan and Medany, 2014).

The oyster mushrooms can grow from a wide variety of agricultural and forestry wastes, such as rice hulls, wheat straw, corn cobs, sawdust, and a variety of other wastes, which has led to its use as one of the eco-friendly solutions for converting wastes into the biomass of high market value, with the potential for reuse of the mushroom substrate in either mushroom production, farming land as organic fertilizer's to maximize yields, as an animal feed and as a biogas production while minimizing eutrophication.

Zn plays a vital role in many physiological activities among all the essential micronutrients. Zinc is a trace element for all living things (White and Broadley, 2005; Graham *et al*., 2007). It is one of the most important minerals needed by our body systems due to the fact that it is highly associated with protein and carbohydrate-rich foods. It is also used in medicines that treat rashes, acne, dandruff and athlete's foot (Okwulehie and Ogoke, 2013).

Zinc is a powerful antioxidant because it is a cofactor for superoxide dismutase and other enzymes. It helps to maintain the molecular structure of subcellular organelles and their membranes. It is required to metabolize nucleic acids, proteins, carbohydrates, lipids, and secondary metabolites, which impact cell division, growth and repair (Plum *et al*., 2010). Three hundred enzymes and 1000b transcription factors require zinc for their function, zinc interacts with ligands and plays an important role in the metabolism of RNA, DNA, signal transduction and gene expressions (Cherasse and Urade, 2017). Formation of secondary metabolites, preventing morphological distortion of protoplasts and spheroplasts, the interaction of cell wall components to anchor lipopolysaccharides, and stabilizing membrane for all these activities zinc is essential (Chapman, 1994).

According to recent estimates, half of the world's population may be deficient in Zn. The amount of Zn in natural (unfertilized and uncontaminated) soil is related to the parent rock's chemical composition and the extent of weathering processes (Chesworth, 1991). Zn deficiency is more common in calcareous (high pH) soils that machines have levelled for uniform irrigation application. Because field levelling, particularly in calcareous soils, destroys topsoil and organic matter rich in micronutrients.

Several studies have demonstrated that poor soil Zn availability, rather than a lack of total Zn, is the primary cause of widespread Zn shortage in cereal crops (Zou *et al*., 2012). Organic matter is essential in regulating soil Zn availability (Chami *et al*., 2013). The maturity of organic inputs determines the impact of organic matter on soil-Zn availability. To improve Zn availability in soil, the application of zn fertilizer is most important (Modaihsh *et al*., 1990 and Ma *et al.*, 2006). In addition to manure and compost, crop straw is aggressively encouraged and widely used as an acceptable and environmentally beneficial organic amendment worldwide. Zn deficiency is a significant health problem in many countries, especially in regions where people rely on cereal-based food with small Zn concentrations (Cakmak *et al.*, 2018).

In humans, zinc is required for average cell growth and development, DNA synthesis, neurosensory functioning, and cell-mediated immunity. Although elderly persons consume less zinc, its inadequacy and effects on cell-mediated immunity have not been shown. Essentially Zinc in humans was established in humans during 1961 (King *et al*., 2006). The recommended dietary allowance is 15 mg/day. An adult human's total body zinc is roughly 1.5–2.0 grams or

about 0.003 % of total weight. Adult males require 1.4 mg of zinc per day, while females require only 1.0 mg per day (WHO 1996).

Zinc is present in meat, liver, kidney, fish, poultry, and grains, and it is best consumed with vegetables. Zinc is present in traditional stable foods such as grains, legumes, and tubers, but its bioavailability is reduced due to phytate, fibre, and lignin. Zinc content in plants fluctuates depending on zinc levels in the soil. Wheat (germ and bran) and different seeds are the food plants that contain the most significant zinc when there is enough zinc in the ground (sesame, poppy, alfalfa, celery and mustard). Beans, nuts, almonds, whole grains, pumpkin seeds, sunflower seeds, and blackcurrant contain zinc (Ensminger *et al.,* 1993). Due to restricted access to zinc-rich foods (animal products, oysters, and shellfish) and the number of zinc inhibitors, such as phytates, found in plant-based diets, millions of individuals worldwide may be deficient in zinc (Sandstead *et al.,* 1991). Coffee contains tannin, reducing zinc absorption (Wikoff *et al.*, 1993).

Nowadays, zinc deficiency is a global problem, causing a severe threat to crop production and human health (Farooq *et al*., 2018). It is more common in high cereal intake locations and low animal food consumption (Roohani *et al*., 2013). Although the diet may not be deficient in zinc, its bioavailability is crucial to its absorption. Zinc is known to be inhibited by phytic acid. Infants, children, adolescents, pregnant, and lactating women have higher zinc requirements than adults, putting them in danger of zinc deficiency. Growth failure occurs when zinc levels are low during growth periods. Zinc deficiency has the most clinical impact on the epidermis, gastrointestinal, central neurological, immunological, skeletal, and reproductive systems, it also results in acne, eczema, lowers protein level in the prostate and testes, dwarfism, impaired learning ability and various types of dermatological problems (Falla *et al.,* 2018). The diagnosis of marginal Zn insufficiency in humans is still tricky. Zn deficiency also reduces antioxidant capacity, supplementation with these elements improved antioxidant status in animal tests significantly (Yan *et al*., 2012 and Chang *et al*., 2004). Immunosuppression slowed physical growth and cognitive function, and various dermatological issues are also symptoms of zinc insufficiency. Dietary modification, supplementation, fortification, and biofortification are the four main intervention options for preventing zinc insufficiency.

High-dose zinc supplementation leads to zinc toxicity, which can cause reduced immunological response, hypocupremia, microcytosis, and neutropenia. Acute and chronic zinc

poisoning are also possible. Nausea, loss of appetite, vomiting, abdominal cramps, diarrhoea, and headache are some of the acute side effects of high zinc consumption. It may also result in ataxia and lethargy. Low copper status, altered iron function, impaired immune function, and reduced high-density lipids lipoproteins have been linked to daily zinc intakes of 150–450 mg (Hooper *et al*., 1980).

Zinc enrichment can be done by adding Zinc oxides (ZnO) and zinc sulphates (ZnSO4H2O or ZnSO4.7H2O) which are the most common zinc micronutrients for fertilizer fortification. Many earlier studies (Dimkpa *et al*., 2017; Du *et al*., 2019 and Subbaiah *et al*., 2016) found that applying Zn boosted grain production. Several studies have suggested that nanoparticles positively affect the overall germination, growth and performance of different crops. Seed germination, seedling growth, photosynthetic efficiency, biomass, total protein, sugar, nitrogen and micronutrients have been efficiently increased in several crop plants; *viz*., *Spinacia oleracea* (Srivastava *et al*., 2014), *Cucumis sativus* (Moghaddasi *et al*., 2017), *Solanumly copersicum* (Faizan *et al*., 2018), and *Triticum aestivum* (Zhang *et al*., 2018). Moghaddasi *et al*., (2017) reported that *C. sativus* grown in a gel chamber showed increased shoot and root biomass with ZnONPs (1 mg/L) and increased shoot length with ZnONPs compared to bulk ZnO.

Almost 50% of the international agricultural soil is deficient in available Zn, and approximately 30% of the global population is subjected to Zn deficiency, especially in developing countries (Kopittke *et al*., 2019 Phattarakul *et al*., 2012). To overcome this problem, many researchers have worked on enhancing zinc content by various means like enrichment of the soil, substrate where the mushroom is grown etc.,

Enhancement of crop yield with the application of zinc sulphate nanoparticles has been reported by Du *et al.,* (2019). According to them, the treatment of ZnO nanoparticles increases the yield and biomass of wheat by 56 & 63 percent and 55 and 72 percent, respectively, compared to control. After the zinc oxide nanoparticle treatment in coffee plants, the fresh weight of leaves and roots was increased by 37 percent and 95 percent, respectively, compared to control. ZnO nanoparticles application enhanced NPK content in rice, with subsequence increasing panicle number (3.8–10.3%), spikelet number per panicle (2.2–4.7%), and total biomass (6.8–7.6%), thereby promoting the rice yield. Compared with conventional fertilization (Yang *et al*., 2021).

Mushroom substrate is enriched with various micronutrients by researchers to enhance their microelements. As the fungal mycelium is good at absorbing from the substrate and storing them, the enrichment of micronutrients is easy than other ways of enrichment. Hence several growth strategies have been effectively used to boost the nutritional content of mushrooms (Estrada *et al*., 2013 and Bhatia *et al.*, 2014). The enrichment with micronutrients impacted antioxidant activities and phenolic content (Bhatia *et al.*, 2014; Vieira *et al*., 2013 and Gąsecka *et al.*, 2015).

Biofortification increases the micronutrient content through selective breeding, genetic modification, or enriched fertilizers. Biofortification of mushrooms is being researched to reduce micronutrient deficiency. Mycelium of *P.eryngii* is distinguished by its ability to absorb bio-elements from growing media, allowing it to be biofortified with trace components with functional activity in the human body.

Consumption of antioxidant-rich foods protects human health by reducing oxidative damage and increasing the formation of free radicals (Oboh *et al*., (2012); Graversen *et al.*, (2008)). Free radicals are unstable and highly reactive due to having unpaired electrons; they are responsible for oxidative stress, and in consequence, they cause DNA damage, carcinogenesis, oxidation of biomolecules and cellular degradation related to ageing, etc. (Hu *et al*., (2002); Prasad *et al*., (2004); Willcox *et al*., (2004); Valko *et al*., (2006)).

Many studies have been conducted to discover and develop natural antioxidants. It is believed that antioxidants and compounds with radical scavenging activity protect against free radical damage. Among other naturally occurring substances, mushrooms may be one of the valuable candidates to search for an effective antioxidant with radical scavenging activity (Liu *et al*., 1997). Phenolic chemicals are potent radical scavengers, free radical inhibitors, and phytonutrients found in mushrooms (Michalak *et al.*, (2006) and Newell *et al*., 2010).

The interest in natural antioxidants and their uses in producing a new generation of food has increased recently. The intake of an exogenous antioxidant is critical to maintaining adequate antioxidants to ensure balance with the prevention of various diseases (cardiovascular disease, type 2 diabetes mellitus, reproductive pathology and several common cancers). Special attention is paid in this context to the mushrooms. Many biologically active substances are of interest as valuable raw materials for functional products, especially those with an antioxidant effect. Oyster mushrooms are promising because they are readily available and rich in natural antioxidants.

Oyster mushrooms contain much more sulfur-containing antioxidants than other regularly consumed mushrooms, such as white button, crimini, or portabella mushrooms (Dubost *et al*., (2006); Kalaras *et al.,* (2017)).

A variety of treatments were used to boost dietary Zn consumption (Stein, 2010). They include genetic and agronomic biofortification of an edible crop (Graham *et al*., (1999); White and Broadley, (2005, 2009)); Cakmak, 2008; Khoshgoftarmanesh *et al*., (2009); Bouis and Welch, (2010); Martínez-Ballesta *et al*., (2010)). Increased soil Zn phytoavailability or Zn fertilizers can help with agronomic biofortification. This requires the proper infrastructure, but it can be pretty successful in areas where mineral fertilizers boost crop yields, and Zn is added at the manufacturing or distribution stage (Cakmak, 2004, 2009). Increased Zn acquisition from the soil and accumulation in edible portions is the basis for genetic biofortification. There is enough Zn to generate biofortified crops for several years in most agricultural soils if it becomes photo available (Graham et al., 1999). Most economic assessments show that genetic techniques for Zn biofortification are more practicable, long-lasting, and cost-effective than dietary diversification, supplementation, or food fortification initiatives for improving Zn intakes in vulnerable populations (Horton, 2006).

Mushrooms can be biofortified by selenium, zinc, magnesium, copper, iron etc. therefore, the fortified mushroom has some beneficial antioxidant effects. In order to enhance zinc, the essential trace elements ZnSo4 nanoparticles at 10 ppm help in enhancing the yield and biological efficiency of *Pleurotus florida.* Moreover, this will help in the biofortification of zinc in these mushrooms and, in turn, help alleviate zinc deficiency and ensure food and nutrition security in the country (Kushwaha *et al*., 2021).

P. eryngii has been discovered to be a rich source of significant major minerals such as K, Mg, Ca, Fe, and a few minor minerals. Enriching growing media with Zn and Se salts increases the quantity of these metals in the mycelium and fruiting bodies, with a variety of effects on other chemical constituents. We can create *P. eryngii* fruiting bodies or mycelium with predictable levels of Zn and Se by carefully selecting the dose, form, and method of application (Zieba *et al.,* 2020). Therefore, keeping the above facts in mind present investigation is carried out on the biofortification of *Pleurotus* mushroom using zinc sulphate.

Materials and Methods

Materials and Methods

The present study was conducted to find the amount of zinc sulphate needed to enrich the Zinc content in three edible *Pleurotus* sp. and to analyse the impact of enrichment on Antioxidant activity.

3.1 Collection of materials

1. ***Substrate***: Paddy straw was used as substrate. It was collected from TNAU, Wetland, Poosaripalayam, Coimbatore
2. ***Spawn***: Spawn of the *Pleurotus florida*, *Pleurotus eous* var.APK1 and *Pleurotus ulmarius* var.CO2 was collected from Mushroom Cultivation Center, TNAU, Coimbatore

3.2 Substrate preparation

Paddy straws were chopped to 4 cms length and soaked in water for 2 hrs. The soaked straws were water drained and sterilized in an autoclave. After sterilization, they are bagged in heat-resistant polythene bags after reaching room temperature.

3.2.1 Pasteurization of substrate for cultivation:

The pasteurization of substrates viz., boiling, streaming, and autoclaving pre-soaked paddy straw for 30 minutes at 15 psi. Three replications were maintained for each treatment and the observations were recorded: number of days taken for spawn run, button formation, total yield, and biological efficiency.

3.3 Cultivation and Harvesting of Mushroom

Mushrooms were cultivated from the mushroom hut, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore with zinc treatment (Table 2).

3.3.1 Sanitation of incubation and cropping room:

The incubation and cropping rooms were cleaned and disinfected by spraying 0.2 % carbendazim. After spraying, the room was kept airtight for 24 hours and then opened for fresh air circulation.

3.3.2 Cultivation

- Sterile chopped paddy straw was bagged in the transparent polythene bags of 60 x 30cm size with a thickness of 100 gauge.

Table:3 Treatments for Zinc enrichment

Treatments	Zn Treatment details
Without Zinc	Control
1.5% Zn	1.5% Zinc Sulphate Per 500gm of the dry weight of the substrate
2.5% Zn	2.5% Zinc Sulphate Per 500gm of the dry weight of the substrate

- Cylindrical beds were prepared following the layer spawning method described by Sivaprakasan (1980).
- A layer of paddy straw was placed at the bottom of the polythene bag, over this, 20 gm of spawns were sprinkled.
- Five layers like the above-mentioned way were bagged and then the bag was tied at the top (modified cylindrical bed method).
- Eight holes of 1 cm diameter were made at random in the polythene bags.
- The mushroom beds were hanged from the bamboo using ropes (‘uri’method).
- After the spawn running stage, the temperature was maintained at 23 to 28° C and relative humidity at 80to 90 %.
- Water was sprinkled regularly as in the std cylindrical bed preparation method.
- The moisture of the mushroom hut was maintained at 80-85% relative humidity by spraying water three times per day.
- The temperature of the mushroom hut was maintained between 22 and 25° C while primordia starts to emerge.
- The number of primordia (pinhead-like appearance) was counted and recorded.
- Oyster mushrooms achieved maturity within two or three days after primordial initiation.

3.3.3 Harvesting

- The matured fruiting body was identified by the curve margin of the cap, as described by Amin *et al*., (2007).

Figure 1
Pleurotus sp. used in this study

Pleurotus florida

***Pleurotus eous* APK1**

***Pleurotus ulmarius* CO2**

- Mushrooms were harvested by twisting to uproot from the base.
- Fully developed fruiting bodies were counted to determine the number of effective ones; tiny and deformed fruiting bodies were discarded at the time of counting.
- To obtain the average weight of individual fruiting bodies, the weight of each flush was taken and divided by the number of fruiting bodies.
- The experiment was a completely randomized design, with replicates.
- The data were subjected to analysis of variance and the mean was compared by Tukey's test ($p<0.05$)

3.4 Mushroom Quality analysis

3.4.1 Morphological study

3.4.1.1 Diameter of the pileus

Matured fruiting bodies were taken to collect the data. Pileus diameter was measured using a metric scale (cm)

3.4.1.2 Thickness of fruiting body

Cap from the mature fruiting body was taken and the thickness of the cap was observed both in the center and the edges using a micrometer screw gauge (mm). The average of both was calculated as the thickness of the fruiting body.

3.4.2 Yield

The yield was calculated with the formula

$$\mathbf{Y = FW\ (g)\ x\ S}$$

Where

FW = Fresh Weight of mushroom

S = Substrate fresh weight (Kg)

3.4.3 Biological efficiency

The biological efficiency (BE) was calculated according to Wang, Sakoda, and Suzuki (2001):

$$\mathbf{BE\ \%} = \frac{\mathbf{fresh\ weight\ of\ harvested\ mushroom}}{\mathbf{dry\ weight\ of\ the\ substrate}} \mathbf{x\ 100}$$

3.4.4 Determination of moisture and dry matter

Moisture amount was determined by keeping the weighed quantity of sample in a thermostat-controlled oven at 105ºC for 6 hours.23-24 The dry weight of each sample was taken on an electric balance. The percentage of the moisture content and dry matter was then calculated by the following formula:

$$\textbf{Moisture}\ (\%) = \frac{\textbf{Initial Wt} - \textbf{Final Wt}}{\textbf{Original weight of sample}} \textbf{x 100}$$

$$\textbf{Dry matter}\ (\%) = \textbf{100} - \textbf{Moisture}\ (\%)$$

3.4.5 Ash Content

- ✓ A 2 g sample from the finely ground mushroom was placed in a crucible and converted into ash at 550 - 600°C for 5 h in a carbonite muffle furnace after which it was allowed to cool in a desiccator.
- ✓ The difference in the weight of the crucible without the sample before and after ashing was used to calculate the ash content.

3.5. Phytochemical analysis

Mushrooms growing from the spawn were collected in packets and all the wastes and dust were removed from the fruiting body. The proximate analysis of the mushroom in the total experiment was conducted with the determination of moisture, total carbohydrate, protein, and also their antioxidant activity using DPPH analysis.

3.5.1 Ethanol extract preparation (Kokate, 1994)

- ✓ About 5 g of powdered material was weighed and macerated with 100 ml of 90% ethanol in a closed flask for 24 hours shaking frequently during the first 6 hours and kept undisturbed for 18 hours.
- ✓ Thereafter, it was filtered rapidly taking precautions against loss of the solvent.

3.5.2 Determination of solubility percentage (Kokate, 1994)

Ethanol

- ✓ About 5 g of powdered material was weighed and macerated with 100 ml of 90% methanol in a closed flask for 24 hours shaking frequently during the first 6 hours and kept undisturbed for 18 hours.

- ✓ Thereafter, it was filtered rapidly taking precautions against loss of the solvent.
- ✓ About 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed, swallowed dish dried at 105˚ C for 6 hrs and cooled in a desiccator and weighed.
- ✓ The content of extractable matter (% w/w) air-dried material was calculated as follows.

$$\textbf{soluble extractives (\% w/w)} = \frac{\textbf{(Weight of residue) x 5 x 100}}{\textbf{Weight of the sample}}$$

3.5.3 Qualitative analysis

3.5.3.1 Test for Carbohydrates

Molisch's test:

To a small amount of the extract, few drops of Molisch's reagent were added followed by the addition of conc. H_2SO_4 along the sides of the test tube. The mixture was then allowed to stand for 2 min and then diluted with 5 ml of distilled water. The formation of a red or dull violet colour at the interphase of two layers indicates the presence of carbohydrates.

3.5.3.2 Test for Proteins

Millon's test:

Extract (3 ml) was mixed with 5 ml of Millon's reagent. A white precipitate is formed. On warming precipitate turn's brick red or the precipitate dissolves giving red coloured solution.

3.5.3.3 Test for Alkaloids

Mayer's test:

The sample (2 ml) was treated with a few drops of Mayer's reagent. The appearance of white precipitate indicated the presence of alkaloids.

3.5.3.4 Test for Glycosides:

Legal's test:

The sample extract was mixed with a few drops of pyridine and 2 drops of 2 % sodium nitroprusside were added. To the reaction mixture, 0.5 ml of 20 % sodium hydroxide was added. The appearance of pink to the red color indicated the presence of glycosides.

3.5.3.5 Test for Saponin

To 1 ml of the extract 5 ml distilled water was added and shaken vigorously. The formation of foam indicated the presence of saponins.

3.5.3.6 Test for Triterpenoids

Extract (5 ml) was dissolved in chloroform (2 mL) and then acetic anhydride (1 mL) was added to it. Concentrated sulphuric acid (1 mL) was added to the solution. The formation of reddish-violet colour shows the presence of triterpenoids.

3.5.3.7 Test for Steroids

To the extract, 2 ml of acetic anhydride and sulphuric acid were added gently by the sidewalls of the test tube and the colour change from violet or blue-green was observed, which indicates the presence of steroids.

3.5.3.8 Test for Tannins

Gelatin test: To a 1ml of gelatin solution, add little 10% sodium chloride. If a 1% solution of tannin is added to a gelatin solution, tannin causes precipitation of gelatin from the solution.

3.5.3.9 Test for Anthroquinone

Bontrager's test

Extract (0.5 ml) was added with 5-10 ml of dilute hydrochloric acid and boiled in a water bath for 10 minutes. The solution was filtered and the filtrate was extracted with benzene and mixed with ammonia solution. The red color was obtained in the ammonia layer that indicated the presence of anthraquinone glycosides.

3.5.3.10 Test for Phenol

Extract (2 ml) was treated with 3ml of 10 % lead acetate. The formation of a precipitate indicates the presence of phenols.

3.5.3.11 Test for Flavonoids

Flavanones:

Sample extract (1 ml) was taken and 10 % of sodium hydroxide was added. Yellow to orange colour formation indicates the presence of flavanones.

Sample extract (1ml) was taken and a few drops of conc.H_2SO_4 were added. Orange to crimson colour formation indicates the presence of flavanones.

3.6 Quantitative test

The three main macronutrients were analyzed

- ✶ Carbohydrates
- ✶ Proteins
- ✶ Fat

3.6.1 Macronutrients:

3.6.1.1 Total Carbohydrates: (Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In a hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone, a green coloured product with an absorption maximum at 630nm.

Materials

- ➢ 2.5N HCl
- ➢ Anthrone reagent: Dissolve 200 mg anthrone in 100 ml of ice-cold 95 % H_2SO_4 prepared fresh before use.

Standard glucose (Stock): Dissolved 100 mg in 100 ml of water.

Working standard – 10 ml of a stock solution was diluted to 100 ml of distilled water. After adding a few drops of toluene, it is stored in the refrigerator.

Procedure

- ✓ About 100 mg of the sample was taken in a boiling tube and it was hydrolyzed by keeping it in a boiling water bath for three hours with 5ml of 2.5N HCl and cooled to room temperature.
- ✓ Then it was neutralized with solid sodium carbonate until the effervescence was created.
- ✓ The volume was made up to 100 ml and centrifuged.
- ✓ The supernatant was collected and 0.5 ml and 1 ml aliquots were taken for analysis.
- ✓ The standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and '0' served as blank.
- ✓ The volume was made up to 1 ml in all the tubes including the sample test tubes by adding distilled water.

- ✓ Then, 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath.
- ✓ Then it was cooled rapidly and the green colour developed was read at 630 nm.
- ✓ A standard graph was drawn by plotting the concentration of the standard on the X-axis versus absorbance on the Y-axis.
- ✓ The amount of carbohydrates present in the sample tube was calculated from the graph.

Calculation

The amount of carbohydrates present in 100mg of the sample is calculated by

$$\frac{\text{mg of glucose}}{\text{volume of test sample}} \times 100$$

3.6.1.2 Estimation of protein (Lowry *et al*., 1951)

Principle

The blue colour developed by phosphomolybdic phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate is measured in the Lowry's method.

Materials:

- ➢ Reagent A - 2 % sodium carbonate in 0.1 sodium hydroxide
- ➢ Reagent B - 0.5 % copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartrate.
- ➢ Reagent C - Alkaline copper solution : Mixed 50 ml of A and 1 ml of B before use.
- ➢ Reagent D - Folin-Ciocalteau Reagent.

Stock standard : 50 mg of bovine serum albumin (Fraction V) was weighed and dissolved in distilled water and the volume was made up to 50 ml in a standard flask.

Working standard: About 10ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg of proteins.

Procedure

Extraction of protein from the sample

Extraction is usually carried out with buffers used for the enzyme assay.

About 50mg of the weighed sample was ground well with pestle and mortar in 5- 10 ml of the buffered centrifuged. The sample was used for protein estimation.

Estimation of protein

- ✓ About 0.2, 0.4, 0.6, 0.8, and 1 ml of working standard were pipette into a series of test tubes and 0.1 ml and 0.2 ml of the sample extract were in two other test tubes.
- ✓ The volume was made up to 1 ml in all test tubes. A tube with 1 ml of water severed as the blank.
- ✓ About 5 ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 minutes.
- ✓ Then 0.5 ml of reagent D was added. Mixed well and incubated at room temperature in the dark for 30 min. The blue colour developed was read at 660 nm.
- ✓ A standard graph was drawn and the amount of protein present in the sample was calculated.

Calculation

The amount of protein present in the sample was expressed in

$$\text{mg/g or 100g} = \frac{\text{mg of protein}}{\text{volume of the test standard}} \times \text{concentration of the standard}$$

3.6.1.3 Estimation of Fat:

Procedure

- ✓ 10 g (W_1) of the mushroom dry sample was weighed accurately, packed well, and placed inside a thimble and a cotton plug was kept on top of it
- ✓ The flat-bottomed flask was weighed (W_2) and then ½ volume of Ether was added to it.
- ✓ The thimble was attached to a Soxhlet apparatus and distilled for 8 hours.
- ✓ The solvent was removed after extraction and evaporated and weighed (W_3).
- ✓ The apparatus was cooled and the solvent was filtered.
- ✓ The flask is then rinsed with small quantities of Ether and then added washings to the above flask.
- ✓ Ether was removed by evaporation and the flask was dried at 80 – 100 °C and then weighed (W_3) (Mishra, 2017).

Calculation

$$\textbf{Fat content } (\%) = \frac{\mathbf{W3 - W2}}{\boldsymbol{W1}} \boldsymbol{X}\ \mathbf{100}$$

Where,

W_1- Weight of dry matter taken for extraction

W_2- Weight of flask before extraction
W_3- Weight of flask after extraction.

3.6.1.4 Determination of Energy (Sade, 2009)

Energy is determined by the given formula

Energy (Kcal) = (Protein value x 4) + (Carbohydrate x 4) + (Fat x 9)

3.6.2 Phytochemical constituents

- ✶ Total Phenol
- ✶ Tannin
- ✶ Flavonoid
- ✶ Vitamin C

3.6.2.1 Estimation of Total Phenol content (Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which can be estimated spectrophotometrically at 650 nm.

Materials

- Ethanol (80 %)
- Folin-ciocalteau reagent
- Na_2CO_3 (20 %)

Stock standard: Gallic acid (100µg/ml in water)
Working standard: Dilute 10 times of stock

Procedure

- Grind 0.5 g of the sample with a pestle and mortar in 10 times the volume of 80 % ethanol.
- Centrifuge the homogenate at 10,000 rpm for 20 minutes. Re-extraction is done and the supernatants were pooled and evaporated to dryness.
- Dissolve the residue in a known volume of distilled water (5 ml).
- Pipette out different aliquots (0.2 to 2 ml) into test tubes.
- Make up the volume in each tube to 3 ml with distilled water.

- Add 0.5ml of Folin-Ciocalteau reagent.
- After 3 minutes, add 2 ml of 20 percent Na_2CO_3 solution to each tube.
- Mix thoroughly, place the tube in boiling water for exactly 1 minute, cool and measure the absorbance at 650nm against a reagent blank.
- Prepare a standard curve using different concentrations of gallic acid.

Calculation

From the standard curve the concentration of phenols in the sample was observed and expressed as GAE mg of phenols/g of material.

3.6.2.2 Estimation of Tannin (Folin and Ciocalteu Method, 1927)

Principle

The principle behind this method is the reduction of Phosphotungstomolybdic acid in an alkaline solution to produce a coloured complex.

Materials

1. Folin- Ciocalteu reagent
2. Sodium carbonate
3. Tannic acid

Procedure

- To 0.1 ml of the sample extract, 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent, 1 ml of 35% sodium carbonate solution were added and diluted to 10 ml with distilled water.
- The mixture was shaken well, kept at room temperature for 30 min, and was measured at 725 nm.
- The Blank was prepared with water instead of the sample.
- A set of standard solutions of Tannic acid is treated in the same manner as described earlier and read against a blank.

Calculation

The results of Tannin are expressed in terms of Tannic acid in mg/g tissue TAE of extract.

3.6.2.3 Determination of total flavonoid content (Grubesic *et al*., 2005)

Principle

The content of flavonols was determined by using rutin as a reference compound. This method was based on the formation of a complex with maximum absorption at 440 nm.

Reagents:

1. Aluminium chloride solution (20 mg/ml)
2. Sodium acetate (30 mg/ml)

Procedure

- About 1 ml of each extract was mixed with 1 ml aluminium chloride and 3 ml sodium acetate.
- After 2.5 hrs the samples were read at 440 nm absorbance.
- The absorption of standard rutin solution in methanol was measured under the same conditions.
- A duplicate was carried out for all the determinations.

Calculation:

The amount of flavonoids in plant extracts in rutin equivalents were calculated by the following formula.

$$X = (A - m_0) / (A_0 - m)$$

Where,

X = flavonoid content in rutin equivalents

A = absorption of plant extraction solution,

A_0 = absorption of standard rutin solution,

m = weight of plant extract

m_0 = weight of rutin in the solution

3.6.2.4 Determination of Vitamin C (Satpathy *et al.*, 2021)

Materials required

- 1% starch indicator Solution
  - Dissolve 1 gm of soluble starch in 100 ml of near-boiling distilled water.
  - Mix well the above and cool before use
- Iodine solution
  - Dissolve 5 gm of Potassium iodide (KI) and 0.268 gm of Potassium iodate (KIO_3) in 200 ml of distilled water.

- ✶ Add 30 ml of sulfuric acid (3 M) to the above solution.
- ✶ The above mixture is made up of 500 ml of distilled water.

Standard: Dissolve 0.250 gm of Ascorbic acid in 100 ml distilled water. Makeup to 250 ml.

Standardizing Solutions

- ✓ 25 ml of vitamin C standard solution was taken into a 125 ml Erlenmeyer flask, to which 10 drops of 1% starch solution were added.
- ✓ Burette is rinsed well with iodine solution and then filled with the same iodine solution. Record the initial volume.
- ✓ Titrate the solution until the endpoint is reached.
- ✓ The endpoint of the titration is the appearance of blue colour, which persists after 20 seconds of swirling.
- ✓ Record the final volume of iodine solution, which is the volume that was required in the starting volume minus the final volume.
- ✓ Repeat the titration at least twice more. The results should agree within 0.1 ml.

3.6.2.5 Determination of Zinc

Zinc determination is done using Microwave plasma atomic emission spectrometer.

0.5 gram sample volume made upto 50 ml analysis done in MP-AES.

TRIPLE ACID EXTRACT:

- ✓ The organic constituents of the plant are hydrolyzed and converted into soluble inorganic constituents by the action of tri acid mixture. By mixing conc.
- ✓ Nitric acid, sulphuric acid and perchloric acid in 9:2:1 ratio triple acid mixture is prepared.

Procedure:

- ✓ Weight about 1 g of given sample into a 100 ml conical flask.
- ✓ Add 15 ml of the triple acid mixture and cover the mouth of the flask and keep it over a sand bath.
- ✓ Continue heating until the contents is reduced to about 2-3 ml of colourless clear solution.

- ✓ If white residues is not obtained, the residue should be treated again with 2 ml of tri acid mixture and the heating be continued till the contents is reduced to about 2-3 ml. therefore the beaker be removed from the sand bath.
- ✓ Allow the beaker to cool.
- ✓ Add 5 ml of distilled water into the beaker containing digested residue.
- ✓ Heat the contents gently until white fumes appear
- ✓ Remove the beaker and let it cool. Then add 10 ml of distilled water and heat the contents to dissolve the residue.
- ✓ Take 100 ml clean volumetric flask fitted with a funnel lined with whatman no. 42 paper.
- ✓ Transfer the contents of beaker into the funnel using a glass rod collecting the filtrate in the volumetric flask.
- ✓ Rinse the beaker with about 15 ml portions of glass distilled water and transfer each rinsing into the funnel in order to transfer the digested residue quantitatively.
- ✓ Collect the filtrate from each washing into the same volumetric flask.
- ✓ Then wash the residue on the filter paper with small portion of distilled water and collect the washing until the volume of filtrate reaches 100 ml mark.
- ✓ Stopper the flask , label it and use it for further analysis of P, K, Ca and Mg concentration.

3.6.3. Antioxidant activity

3.6.3.1. DPPH Antioxidant Activity

Principle `

DPPH radical reacts with an antioxidant compound that can donate hydrogen and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

Reagents

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

- ✓ The samples were reacted with the stable DPPH radical in a methanol solution.
- ✓ 0.5 mL of sample, is taken and 3 mL of absolute methanol is added to it.

- ✓ 0.3 mL of DPPH radical solution 0.5 mM in methanol is added to the above mixture.
- ✓ When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced.
- ✓ The changes in color (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 100 min of reaction using a UV-VIS spectrophotometer

Calculation

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank})x100}{Abs_{control}}\right]$$

Results and Discussion

Results and Discussion

The present study is to investigate the ability of *P. florida, P. eous* APK1, and *P. ulmarious* CO2 to absorb zinc from the substrate enriched with Zinc Sulphate. Various parameters and antioxidant activity were studied in all the three mushrooms on biofortified and non-biofortified samples to check the impact of biofortification of Zn on growth and a few biochemical constituents.

Mycelial growth was found to decrease at higher concentrations of $ZNSO_4$ treatment at higher concentrations (2.5%). *P. ulmarious* CO2 was most affected at 2.5 % concentration hindering the growth of mycelium resulting in poor yield in all the triplicates, which was visibly recorded. Mycelial growth and pinhead formation of *P. ulmarious* was found to be slow yet resulted in good yield (Joshi *et al*., 2018)

The first flush of harvest occurred between 19 - 35 days of incubation. In *P. florida* the first harvest happened between 22-24 days, and the harvest in *P. eous* APK1 had in 18-23 days, while *P. ulmarious* CO2 took 28 - 35 days. It was recorded that the formation of a pinhead was very less in *P. ulmarius* at 2.5 % concentration. All the mushrooms with and without treatments sprouted in clusters and had fruiting bodies. Bioaccumulation of zinc in various $ZNSO_4$ concentrations does not show any alteration to size, shape appearance, or colour.

Hypsizygus ulmarius CO2 strain and II HR Hu1 strain took 19 - 21 days and 20-22 days for spawn running respectively, while harvest of fruiting body happened in 38-42 days in *H. ulmarious* CO2 strain and 38-43 days in IIHR Hu1 strain (Usha and Suguna, 2016).

Biological Efficiency and Yield

The biological efficiency of *P. florida* (118.03±25.68) was higher than the other two species compared to the non-biofortified mushrooms. The performance of all the three mushrooms was different with regards to the enrichment using $ZNSO_4$. In *P. florida* the biological efficiency was reduced in both the treatments compared with control. as the treatment of $ZNSO_4$ increased. In *P. eous* APK1 the biological efficiency was found to be higher than control in 2.5 % concentration (97.46 %). *P. ulmarious* resulted in lower BE with 2.5 % concentration, but the weight and the size were immense.

Table 4
Evaluation of the three Pleurotus sp for their number of primordias and fruiting bodies

Treatments	**Number of Primordias**			**Number of Fruiting bodies**		
	P. florida	***P. eous* APK1**	***P. ulmarious* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarious* CO2**
Control	18.00 ± 2.65	29.33 ± 12.10	10.67±1.15	128.67 ± 38.00	120.33 ± 6.66	84.00±23.07
1.5 %	11.33 ± 7.51	15.33 ± 2.08	6.00±1.00	87.67 ± 38.85	116.33 ± 9.61	94.67±6.03
2.5 %	14.67 ± 9.02	20.67 ± 6.66	4.00±1.00	65.33 ± 22.85	179.33 ± 91.8	69.67±7.02
	5.3020 14.7209	**6.5715 18.2458**	**0.8607 2.1061**	**29.6229 82.2478**	**46.7962 129.9293**	**0.8607 2.1061**

Values are expressed by mean ± SD of three samples in each group

Table 5
Evaluation of the three Pleurotus sp for biological efficiency

Treatments	**Biological Weight (gms)**			**Economical Weight (gms)**			**BE (%)**		
	P. florida	***P. eous* APK1**	***P. ulmarious* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarious* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarious* CO2**
Control	590.15±128.40	359.92±128.0	389.38±127.4	477.16±10.62	316.7±115.74	345.14±112.15	118.03±25.68	71.97±25.60	77.89±25.51
1.5 %	502.74 ± 60.99	216.12±75.46	523.34.±75.39	354.94±29.59	190.90±69.43	458.73±53.87	100.48±12.09	43.22±15.09	112.69±15.93
2.5 %	197.06 ± 65.12	487.3±103.8	323.92±30.25	162.59±57.14	403±70.14	316.06±47.14	39.40±13.02	97.46±20.76	67.27±12.94
	78.6986 218.5058	**89.6894 249.9293**	**71.2386 174.3223**	**39.2734 109.0422**	**75.6121 209.9361**	**62.7216 153.4811**	**15.6923 43.5694**	**17.9318 49.7876**	

Values are expressed by mean ± SD of three samples in each group

In *P. ulmarious* the BE% at the concentration of 1.5 % didn't show any decrease, which shows resistance or tolerance of the fungus to Zn or the amount of $ZnSo_4$ added to the substrate was not sufficient to cause inhibition or any toxic effect to the fungus. (de Assunção *et al*., 2012)

Our results were near to the findings of Mohapatra and Behera (2013) who found that *P. florida* performed better with regard to BE (115.33%) and *H. ulmarious* (102.83%). The Biological Efficiency of *P. florida, P. eous,* and *H. ulmarious* during the Rabi season (December to February) were recorded as 85 %, 70.4 % and 83 % respectively (Kumar *et al*., 2020). BE of the two strains *H. ulmarious* CO2 strain and IIHR Hu1 strain were 46.22±4.98 % and 38.61±4.30 % respectively (Usha and Suguna, 2016). BE % of *P. florida* and *H. ulmarious* with paddy straw as substrate was recorded as 57.63 % and 90.100 % respectively (Joshi *et al*., 2018). The biological efficiency of *P. ulmarious* on paddy straw was maximum of 68.84 % (Mungekar *et al*., 2013). Biswas & Kuiry (2013) reported that the biological efficiency of *H. ulmarious* was the maximum (156%) followed by *P. florida* (121.1 %) and P. sajor-caju (115.5%). Raina *et al*. (2009) revealed that *H. ulmarious* biological efficiency in two different substrates wwasthat using wheat straw it was 83.65 % and using paddy straw it was 76.6 %.

The biological efficiency of *P. florida, P. sajor-caju* and *P. eous* was observed by Naraian and Dixit (2017) after growing them on cattail weed as substrate was 90%, 89 % and 82 % respectively. Shivani *et al*. (2021) and her coworkers observed that biofortification of Zinc sulphate (10 ppm) nanoparticles on *P. florida* had the highest yield when compared to other concentrations. As the concentrations increased their yield decreased. They also found that BE of *P. florida* for 2 kg of the wet substrate was recorded as 72.50 which increased to 108.83 at 10 ppm and was found to be in a decreasing trend from 20 ppm to 40 ppm as 96.50 to 87.50 respectively (Shivani *et al*., 2021). *P. eryngii* was biofortified with Zinc sulphate and Zinc hydroaspartate, in the culture medium and was found that there was no decrease in the mycelium yield of mushrooms (Zi¸eba *et al*., 2020).

Stipe Girth

The stipe girths in *P. florida* were found to have 5 times increase in 1.5 % concentration (32.43 mm) of $ZnSo_4$ than control (6.49 mm). In *P. eous* APK1 there is an increase in the stipe girth from 3.91 mm to 12.56 mm in control and 2.5 % concentration respectively. In *P. ulmarious*

Figure 2

Graphical representation of primordia present in different concentrations

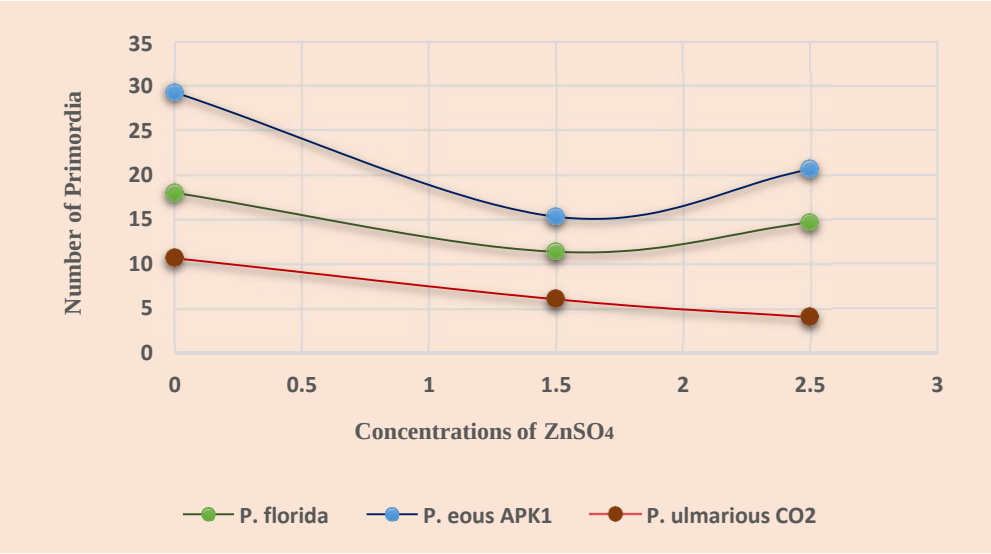


Figure 3

Graphical representation of fruiting bodies in different concentrations

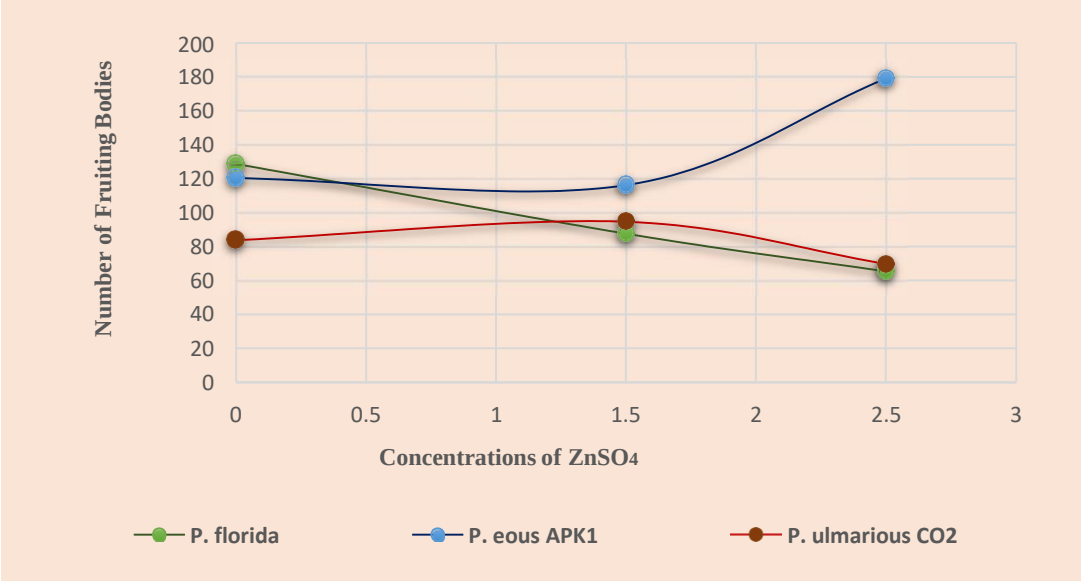


Figure 4

Graph representing the biological and economical weight of mushrooms at different concentrations

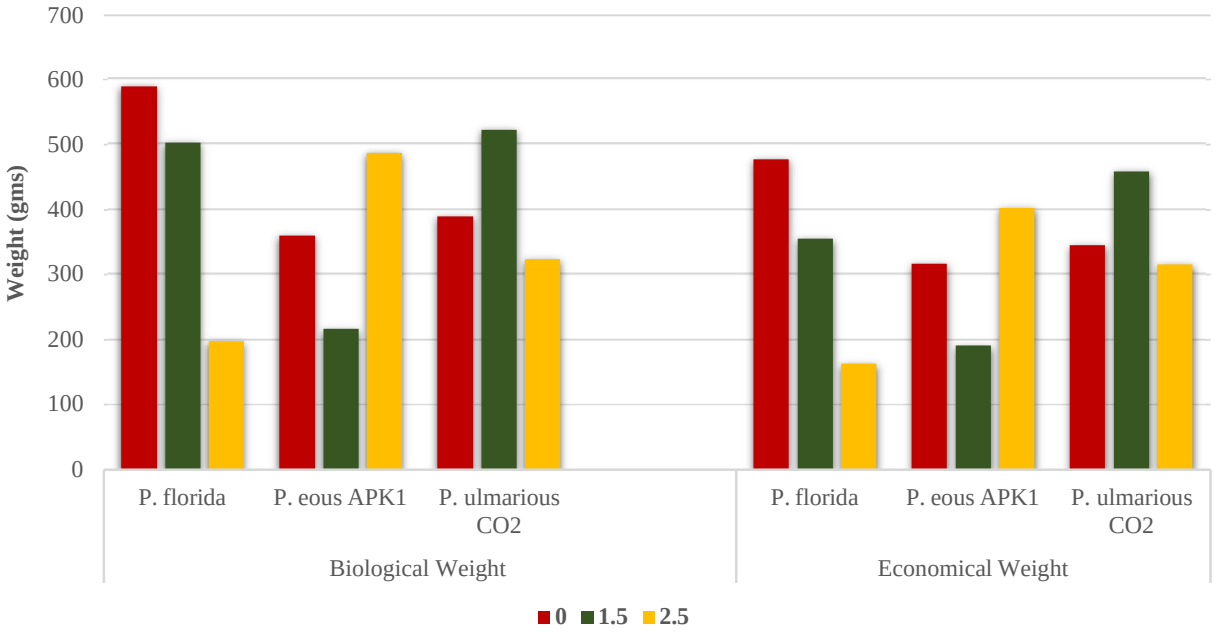
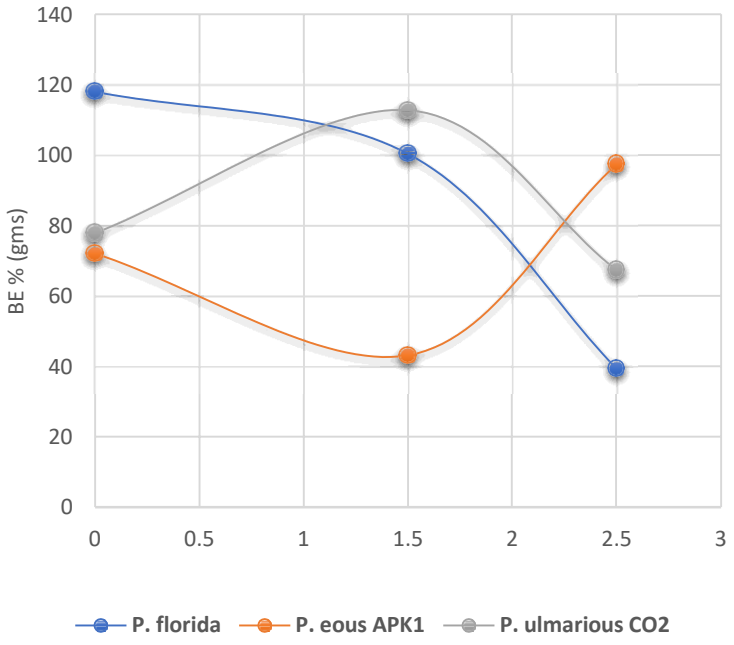


Figure 5

Graph showing the BE % at various concentrations of three Pleurotus sp.



CO2 increase in girth than control was found at 1.5 % concentration (28.19 mm), while at 2.5 % concentration (10.57 mm) the stipe girth has reduced than the control (22.64 mm).

According to Khade *et al*. (2019), the maximum pileus diameter was recorded by the treatment of Karanj cake @ 2% (6.72 cm) followed by the treatment of Rice husk @ 2% (6.55 cm) while the minimum pileus diameter was observed in treatment Soybean flour @ 2% (4.09 cm). And their work revealed a wide variation of stipe diameter ranging from 6.72 to 4.09 cm. Hassan *et al*. (2010) observed the a similar trend of results for *Pleurotus* ostreatus and Mshandete and Kivaisi, (2013) for *P. leurotus* HK-37. According to Praveen *et al*, (2018) *P. florida* had a pileus thickness of 12.62 cm and *P. eous* APK1 had 7.87 cm.

Cap thickness

Cap thickness is found to be maximum in *P. ulmarius* CO2 (3.52 mm) and lowest in *P. eous* APK1 (2.33 mm) in the non-biofortified *Pleurotus* sp. While the biofortified *Pleurotus* species in *P. florida* showed an increase of 1.5 % (3.46 mm) and a slight decrease of 2.5 % (3.32 mm), while in *P. eous* APK1 there is an increase of 1.5 % and 2.5 % from the control which is 2.84 mm and 6.38 mm respectively. Also, in *P. ulmarious* CO2 there is a decrease of 1.5 % (2.79 mm) and a slight decrease (2.88 mm).

Cap thickness was studied on *Pleurotus* ostreatus K22 and P80 strains and *P. pulmonarius* P20 strain by Siwulski *et al*. (2012) resulting in 6 mm, 9 mm and 7 mm respectively.

Yield

The yield of mushrooms is expressed as mg/100g of dry weight. Among the non-biofortified *Pleurotus* sp. *P. florida* had the maximum yield is 1.18 mg/100g, while the minimum yield of 0.72 mg/100g was found in *P. eous* ApK1. Yield after zinc biofortification was analysed and found that the highest (1.00) was found in *P.florida* at 1.5 % and the lowest (0.21) was found in *P. ulmarius* CO2. Though it is to be noted that triplicates of *P.ulmarius* at 2.5 % concentration did not grow well. That can be one of the reasons for the yield reduction in *P. ulmarius* CO2 Findings of Zieba *et al*. (2020) revealed that *P.erynjgii* fortified with Zinc sulfate or Zinc hydroaspartate produced a yield of 12.28 g and 13.97 g dry weight per 100 g−1 of d.w substrate, respectively

Table 6

Effect of different concentrations of zinc sulphate on some yield parameters

Treatments	Pileus Thickness (mm)			Cap Thickness (cm)		
	P. florida	*P. eous* APK1	*P. ulmarius* CO2	*P. florida*	*P. eous* APK1	*P. ulmarius* CO2
Control	6.49	3.91	22.64	2.99	2.33	3.52
1.5 %	32.43	3.21	28.19	3.46	2.84	2.79
2.5 %	8.32	12.56	10.57	3.32	6.38	2.88
SEd **CD (P<0.05)**	**14.3410** **35.0929**	**0.7133** **1.7455**	13.2886 32.5175	**0.1194** **0.2921**	**2.7468** **6.7214**	**0.7850** **1.9208**

Values are expressed by mean ± SD of three samples in each group

Figure 6

Graphical comparison of stipe and cap diameter in the different concentrations of $ZnSO_4$

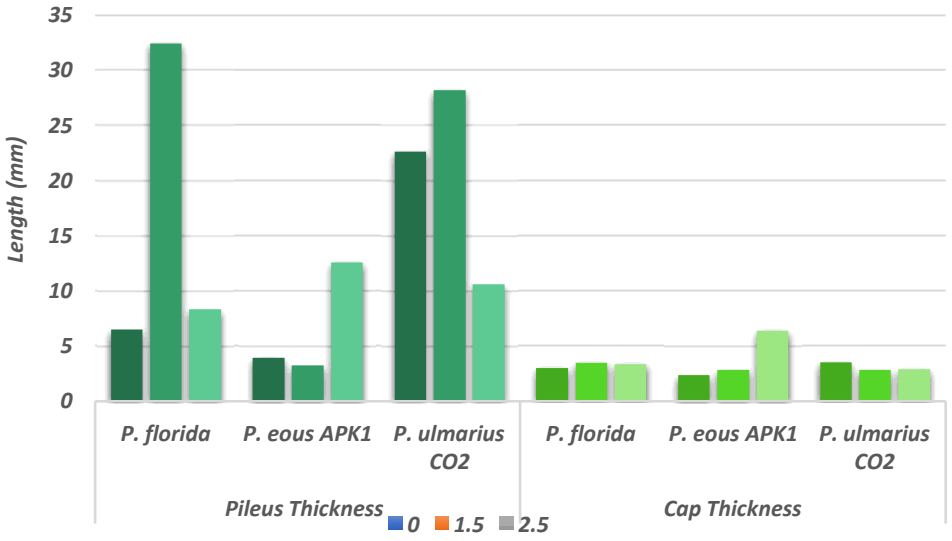


Table:7

Effect of yield at different concentrations of zinc enrichment

Treatments	***P. florida***	***P. eous* APK1**	***P. ulmarius* CO2**
Control	1.18	0.72	0.78
1.5 %	1	0.43	0.37
2.5 %	0.39	0.97	0.21

Table: 8

Moisture content and dry matter of biofortified and non-biofortified Pleurotus sp.

Treatments	**Moisture %**			**Dry matter %**		
	P. florida	***P. eous* APK1**	***P. ulmarius* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarius* CO2**
Control	85.95 ± 0.35	85.73 ± 0.50	80.80 ± 1.74	14.00 ± 0.28	14.27 ± 0.50	19.20 ± 1.74
1.5 %	83.60 ± 1.41	84.67 ± 1.86	81.17 ± 1.11	15.60 ± 2.55	15.33 ± 1.86	21.07 ± 0.97
2.5 %	84.60 ± 1.13	85.13 ± 1.22	84.83 ± 1.36	15.00 ± 1.70	14.87 ± 1.22	18.07 ± 0.81
SEd	**0.9080**	**0.3662**	**0.3506**	**0.9254**	**0.3662**	**0.5319**
CD(P<0.05)	**2.2219**	**1.0166**	**1.7184**	**2.5693**	**1.0166**	**1.4769**

Values are expressed by mean ± SD of three samples in each group

Table: 9

Ash content in Biofortified and non-biofortified

Treatments	***P. florida***	***P. eous* APK1**
Control	0.16	0.17
1.5 %	0.17	0.18
2.5 %	0.14	0.16

Moisture content

Moisture in the substrate is one of the main factors that influence the success of mushroom growth (Bellettini *et al*., 2019). Moisture content and dry matter data are presented in Table 4. The higher moisture content among the three *Pleurotus species* was recorded in *P. ulmarius* CO2 (80.80 %) while the lower value (85.73 %) was observed in *P. eous* APK1. Moisture content was found to be decreasing in 1.5 % of treatment and increasing in 2.5 % but still was lower than the control in *P. florida* and *P. eous*.

The moisture content of various *Pleurotus* species was recorded like *P. florida* had 87.5 % (Amin *et al*., 2018) and 87-88 (Chatterjee *et al*., 2021), *P. ostreatus* had 88.75 % (Tolera and Abera, 2017)

Chang and Miles, (2004) reported that the moisture content of dried mushrooms ranges from 9-13 %, which was also confirmed by Victor and Olatomiva, (2013) by confirming the moisture content of *P. ostreatus* (9% to 10.72%).

Ash content

Ash content estimation in non-biofortified and biofortified mushrooms of *P. florida* and *P. eous* APK1 was done (Table. 7), Since the amount of *P. ulmarius* CO2 produced was very less ash content was not done in that *Pleurotus* species. The ash content in non-biofortified mushrooms was 0.16 and 0.17 in *P. florida* and *P. eous* APK1 respectively. In biofortified mushrooms, both *P. florida* and *P. eous* APK1 showed the highest ash content in a 1.5 % concentration of 0.17 and 0.18 respectively.

P. florida showed to contain 9.02 of ash content in the work done by Ahmed *et al*. (2009). Bora and Kawatra, (2014) worked on the nutritional component analysis and found that in *P. florida* 7.82 per cent of total ash is present. *P. sajor-caju* which was cultivated on rubber, sawdust had an ash content of 1.16 % of fresh weight (Rashidi & Yang, 2016).

Solubility per cent of the solvent methanol was found to have a slight difference in biofortified and non-biofortified *Pleurotus.* When compared within the species, *P. ulmarious* had

Table 10

Ethanol solubility per cent of biofortified and non-biofortified Pleurotus sp.

Treatments	Solubility %		
	P. florida	***P. eous* APK1**	***P. ulmarius* CO2**
Control	13.43 ± 1.14	12.35 ± 1.70	16.71 ± 6.15
1.5 %	13.75 ± 0.31	16.59 ± 6.25	13.78 ± 0.25
2.5 %	13.16 ± 0.35	12.60 ± 0.17	13.30 ± 0.69
SEd **CD (P<0.05)**	**0.6811** **1.8911**	**2.7569** **7.6546**	**2.7283** **7.5752**

Values are expressed by mean ± SD of three samples in each group

Table 11
Phytochemical screening results of three Pleurotus sp.

Test	*P.florida*			*P.eous APK1*			*P.ulmaris CO2*	
	control	**1.5%**	**2.5%**	**control**	**1.5%**	**2.5%**	**control**	**1.5%**
Carbohydrates	+	+	+	+	+	+	+	+
Protein	+	+	+	+	+	+	+	+
Vitamin c	+	+	+	+	+	+	+	+
Alkaloid	+	+	+	+	+	+	+	+
Glycosides	-	-	-	-	-	-	-	-
Saponin	+	+	+	+	+	+	+	+
Triterpenoids	-	-	-	-	-	-	-	-
Steroids	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-
Phenolics	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+

maximum solubility of 17 % and minimum solubility of 12 % in *P. ulmarious* and *P. eous* APK1 respectively.

Phytochemical Screening

A qualitative test was performed on all the three species of *Pleurotus* with their different concentrations of Zinc sulphate applications. The presence of Carbohydrates, Proteins, Vitamin C, alkaloids, Saponin, Steroids, Tannins, Phenolics and Flavonoids were confirmed. Glycosides, triterpenoids, and Anthroquinone were found to be absent. Rahimah *et al*. (2019) worked on *P. ostreatus* and has reported the presence of alkaloids, steroids, flavonoids, saponins, tannins, phenolic components and absence of quinone and triterpenoids, which is par to our findings.

Carbohydrate

The carbohydrates present in the mushroom are pharmacologically very important due to their antitumour and immunomodulating properties. Xylose, rhamnose, glucose, fructose, mannitol, mannose, maltose, sucrose and trehalose are a few forms of carbohydrates which are very important for biological activities (Ferreira *et al*., 2009; Zhang *et al*., 2007). Carbohydrates which constitute the prevailing component of the dry matter of mushrooms usually range between 50 – 60 % (Deepalakshmi & Mirunalini, 2014).

Among the biofortified and non-biofortified samples the difference was not at a huge margin. Carbohydrates in non-biofortified mushrooms resulted in 69.70, 70.60 and 71.09 mg/100g in *P. florida, P. eous* APK1 and *P. ulmarius* CO2 respectively. Though the increase was very minimal the same trend followed with higher carbohydrates in *P. florida* and the lowest value was recorded in *P. ulmarius* CO2.

Carbohydrate present in *P. florida* was 40-43 g/Kg (Chatterjee *et al*., 2021). *Pleurotus* Sp. contains large amounts of carbohydrates ranging between 24.95 and 75.88 % (Patil *et al*., 2010; Koutrotsios *et al*., 2014). Bora and Kawatra, (2014) worked on the nutritional component analysis and found that in *P. florida* 47.80 per cent of carbohydrate is present.

Protein

The increase in the crude protein present in *P. florida* in both the treatments indicates the enrichment of substrate with ZnSO4 did not affect the nutritional quality of *P. florida* but it seemed

Table 12

Effect of different concentrations of zinc enrichment on the nutritional content

Treatments	Carbohydrate			Protein		
	P. florida	***P. eous* APK1**	***P. ulmarius* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarius* CO2**
Control	69.70±0.22	70.60±0.13	71.09±0.13	26.98±2.96	37.22±4.98	28.18±2.49
1.5 %	69.85±0.27	70.54±0.16	71.39±0.24	35.50±0.43	26.76±1.53	25.16±1.62
2.5 %	70.17±0.03	70.71±0.08		41.02±0.65	37.84±0.52	
SEd	**0.2279**	**0.0914**	**0.0475**	**1.1276**	**1.9252**	**2.4662**
CD (P<0.05)	**0.632**	**0.2539**	**0.1319**	**2.7593**	**4.7109**	**6.8475**

Values are expressed by mean ± SD of three samples in each group

Figure 7

Variations in carbohydrates present at different concentrations of $ZnSo_4$

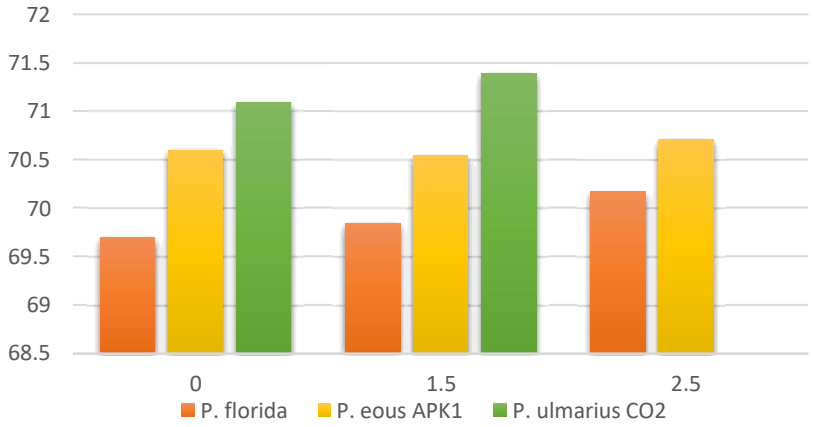
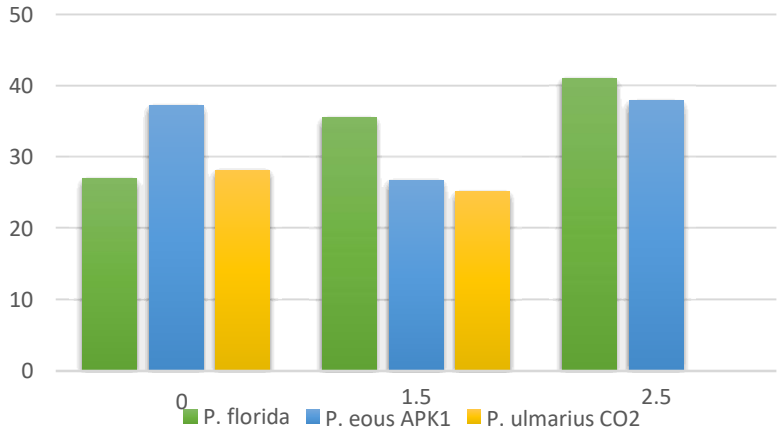


Figure 8

Variations in Protein present at different concentrations of $ZnSo_4$



to affect the crude protein present in *P. eous* APK1 and *P. ulmarious* CO2. In non-biofortified mushrooms, *P. eous* APK1 was found to have maximum (37.22 mg/100g) protein content while *P. florida* had a minimum (26.98 mg/100g) protein content. In biofortified samples of *P. florida* there is a steady increase in protein content (41.02 mg/100 g) and in the biofortified mushrooms of *P. eous* APK1 and *P. ulmarius* CO2, a reduction of protein content from the protein content present in non-biofortified samples was noted

The protein content of dried mushrooms presented in table 11 revealed a common factor among all the biofortified samples of *Pleurotus* were that there is an increase in protein content as the Zn treatment concentration increases. Among the three *Pleurotus* sp. *P. florida* was found to have a higher protein content of (mg/100g) while the lowest value was found in *P. eous* APK1 () without biofortification. The protein content of mushrooms is affected by a number of factors, type of mushroom, stage of development, sampled level of N available and the location (Flegg *et al*., 1977).

Bora and Kawatra, (2014) worked on the nutritional component analysis and found that in *P. florida* 27.92 per cent of crude protein is present. The crude protein content of *P. florida, P. sajor-caju* and *P. eous* were observed by Naraian and Dixit, (2017) after growing them on cattail weed as substrate was 3.8 g, 4.8 g and 3.2 respectively. According to Alam *et al*. (2018) the protein content in *P. florida* was 2.6 g/100 g of fresh mushroom and 20.56 g / 100 g of dried mushroom

Zn biofortification of *P. pulmonarius* has resulted in an increase in protein content (4.2 %) from non-biofortified mushrooms (3.1 %) (Oyetayo *et al*., 2021). The enrichment of substrate with LiCl also did not affect the nutritional quality (de Assunção *et al*., 2012). Proteins of *Pleurotus* spp. are of superior quality, as some species contain complete proteins with a good distribution of essential and non-essential amino acids (Deepalakshmi & Mirunalini, 2014). *P. pulmonaris* without biofortification was found to contain 11.3 mg/100g, while biofortified with zinc showed an increase which was 16.8 mg/100g (Oyetayo *et al*., 2021). The protein present in *P. florida* was 15-21 g/Kg (Chatterjee *et al*., 2021)

Fat

Generally in mushrooms, the fat content present is very low when compared to carbohydrates and proteins. And the fats present in them are dominated by unsaturated fatty acids

Table:13

Effect of different concentrations of zinc enrichment on fat content (g/100 g)

Treatments	*P. florida*	*P. eous* APK1
Control	0.11 ± 0.02	0.11±0.02
1.5 %	0.19 ± 0.02	0.20±0.02
2.5 %	0.28 ±0.01	0.60±0.01
SEd **CD (P<0.05)**	**0.0141** **0.0346**	**0.0141** **0.0346**

Values are expressed by mean ± SD of three samples in each group

Table:14

Effect of different concentrations of zinc enrichment on Energy

Treatments	*P. florida*	*P. eous* APK1
Control	409.83	358.80
1.5 %	443.55	372.80
2.5 %	479.12	420.99

(Singer, 1961). Only because of their low-fat content they are considered low-calorie food. The present finding shows that the fat content in non-biofortified mushroom was the same in all the three *Pleurotus* species, while the biofortified mushrooms showed increase in fat content. *P. eous* APK1 had the highest fat content of 0.60 g/100g at 2.5 % of Zinc sulphate supplementation.

According to Hossain *et al*., 2007 *Pleurotus* species contains fat content from 0.2 to 8 gms per 100 g of dried sample. Oyster mushrooms have been reported to have low fat (Manzi *et al*., 1999; Chirinang & Intarpichet, 2009). Fat content present in *P. pulmonarius* without biofortification and after biofortification with Zn had values of 2.0 mg/100 g and 2.2 mg/ 100 g respectively (Oyetayo *et al*., 2021).

Energy

Energy obtained from non-biofortified *Pleurotus* species were 409.83 and 358.80 in *P. florida* and *P. eous* APK1 respectively. Zinc sulphate biofortification has increased the energy obtained drastically in both the concentrations, in *P. florida* and *P. eous* APK1. *P. florida* was observed to have 443.55 and 479.12 Kcal in 1.5 % and 2.5 % respectively, while *P. eous* APK 1 also showed an increase in both 1.5 % (372.80 kcal) and 2.5 % (420.99 kcal).

Energy obtained from oyster mushroom per 84 g was 27.7 kcal (Fulgoni & Agarwal, 2020).

Total Phenol

Phenolic compounds have been reported to be the major antioxidants determined in mushrooms (Zieba *et al*., 2020). Total phenolic content was found to be 42.76 mg GAE/100g, 30.50 mg GAE/100g and 54.70 mg GAE/100g. *P. florida* and *P. ulmarius* showed an increase at 1.5 % concentration (55.47 mg GAE/100g and 44.43 mg GAE/100g) of Zinc Sulphate supplementation and a decrease in 2.5 % concentration (22.90 mg GAE/100g and 41.64 mg GAE/100g). *P. eous* APK1 had a steady increase at both 1.5 % and 2.5 % concentrations as 41.60 mg GAE/100g and 54.12 mg GAE/100g respectively.

The total phenolic content of the three methanolic extracts was expressed as mg of gallic acid equivalents per gram of mushroom powder. The phenolic compounds could be used as an important indicator of antioxidant activity (Mishra *et al*., 2013). *P. florida* was found to contain 3.72 mg GAE / g of dry matter (Wong *et al*., 2006). Ghosh (2020) and his co-workers reported in

Table 15

Effect of different concentrations of zinc enrichment on phenol and tannin content

Treatments	Total Phenol (mg/100g)			Tannin		
	P. florida	***P. eous* APK1**	***P. ulmarius* CO2**	***P. florida***	***P. eous* APK1**	***P. ulmarius* CO2**
Control	42.76±1.23	30.50±12.51	54.70±6.12	8.89±3.15	6.70±5.04	9.48±3.08
1.5 %	55.47±6.12	41.60±15.91	44.43±9.33	13.26±5.00	6.78±3.31	8.48±1.12
2.5 %	22.90±1.31	54.12±10.63	41.64±0.73	9.19±3.51	3.96±1.25	5.89±0.69
SEd **CD (P<0.05)**	**2.7986** **7.7702**	**12.9898** **36.0660**	**5.6964** **15.8159**	**3.5067** **9.7363**	**3.5444** **9.8411**	**1.6375** **4.5464**

Values are expressed by mean ± SD of three samples in each group

Figure 9

Graphical comparative study of total phenolic content in the three Pleurotus sp.

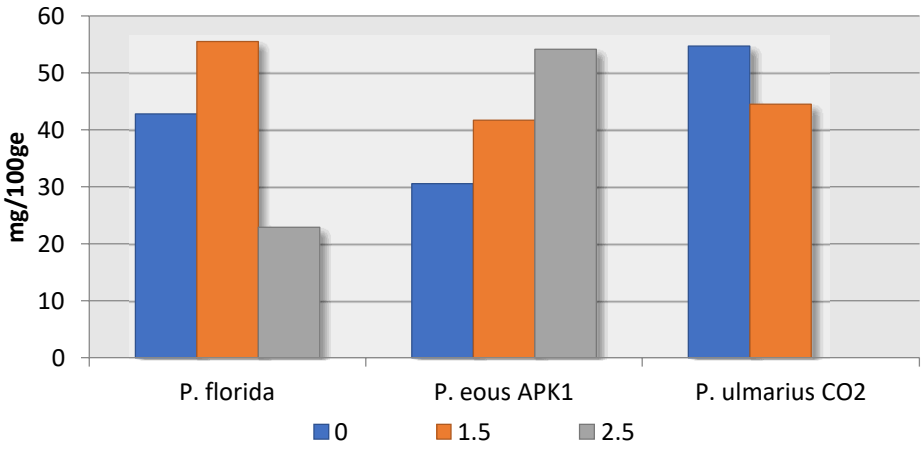
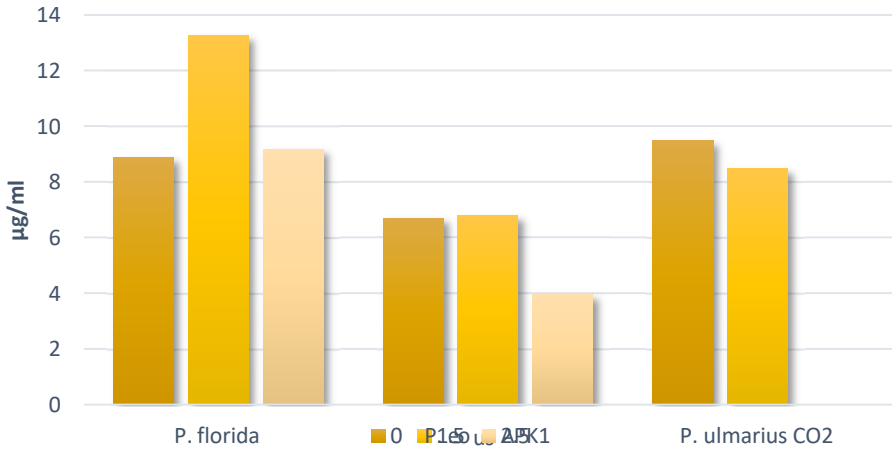


Figure 10

Graphical comparative study of tannin in the three Pleurotus Sp.



their research finding that decoction of P. eous, P. florida and P. ostreatus had 5.78, 2.17 and 5.2 µg GAE/mg of phenol respectively.

Vitamin C

The vitamin C content in non-biofortified *P. florida*, *P. eous* APK1 and *P. ulmarius* CO2 were found to be 1606 µg/ ml, 1785 µg/ ml and 1699.33 µg/ ml respectively. Biofortification at 1.5 % of Zinc Sulphate of *P. florida* yielded the maximum Vitamin C content (1804.67 µg/ ml), while *P. ulmarius* CO2 had minimum Vitamin C content (1783.33 µg/ ml). at 2.5 % concentration also *P. florida* had a maximum of 1843.67 µg/ ml and *P. ulmarius* CO2 had a minimum of 1827.33 µg/ ml of vitamin C.

The presence of Vitamin C is also an indicator that these mushrooms have antioxidant properties (Fu & Shieh, 2001). In *P. florida* 113 g/100 g of dried mushroom were recorded by Banu and Rajarathnam, (1988).

Flavonoids

Flavonoid content when compared between the *Pleurotus* species, *P. florida* was having more (185.67 µg Rutin equivalent/ ml of extract) than the other species. Biofortification has led to a decrease in the 1.5 % concentration of zinc sulphate and an increase in the level in 2.5 % concentration of Zinc sulphate except in *P. eous* APK1 as the flavonoid content present in 2.5 % concentration (70.58 µg Rutin equivalent/ ml of extract) of biofortification did not improve than the flavonoid content present in the non-biofortified sample (83.07 µg Rutin equivalent/ ml of extract).

According to Wong *et* al., 2013 the dry mater of *P. florida* was found to contain 0.17 mg QE / g flavonoids. Flavonoid content in *P. eous* P31 was reported by (Kortei & Kwagyan, 2015) as 311.3 mgQE/g in methanolic extract. Ghosh, (2020) and his co-workers reported in their research finding that decoction of *P. eous, P. florida* and *P. ostreatus* had 2.23, 1.19 and 0.73 µg QE/mg of extract of flavonoid content.

Table 16

Effect of different concentrations of zinc enrichment on Vitamin C

Treatments	*P. florida*	*P. eous* APK1	*P. ulmarius* CO2
Control	1606.00±49.03	1785.00±95.36	1699.33±88.21
1.5 %	1804.67±50.33	1790.00±68.79	1783.33±40.46
2.5 %	1843.67±7.77	1809.00±2.65	1827.33±15.95
SEd **CD (P<0.05)**	**40.7849** **113.2388**	**65.5786** **182.0783**	**48.6918** **135.1922**

Values are expressed by mean ± SD of three samples in each group

Figure 11

Graphical comparative study of Vitamin C

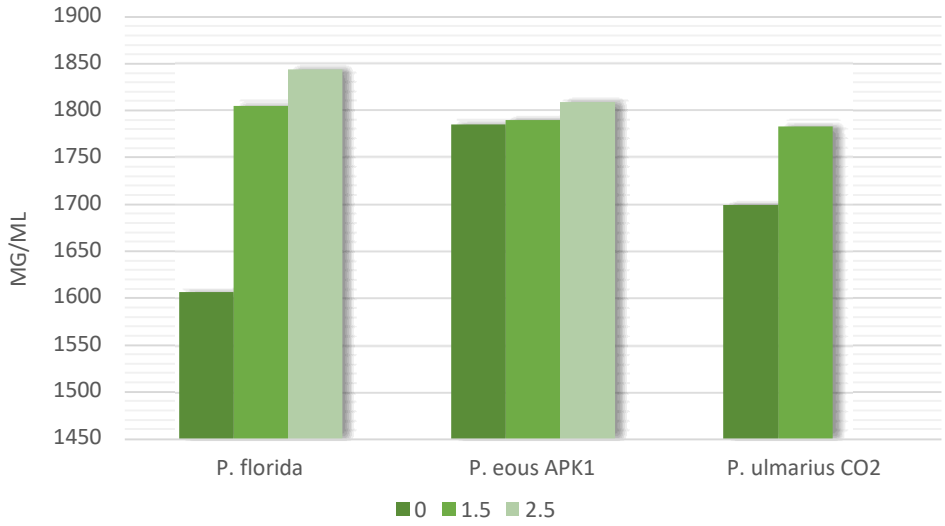


Table 17

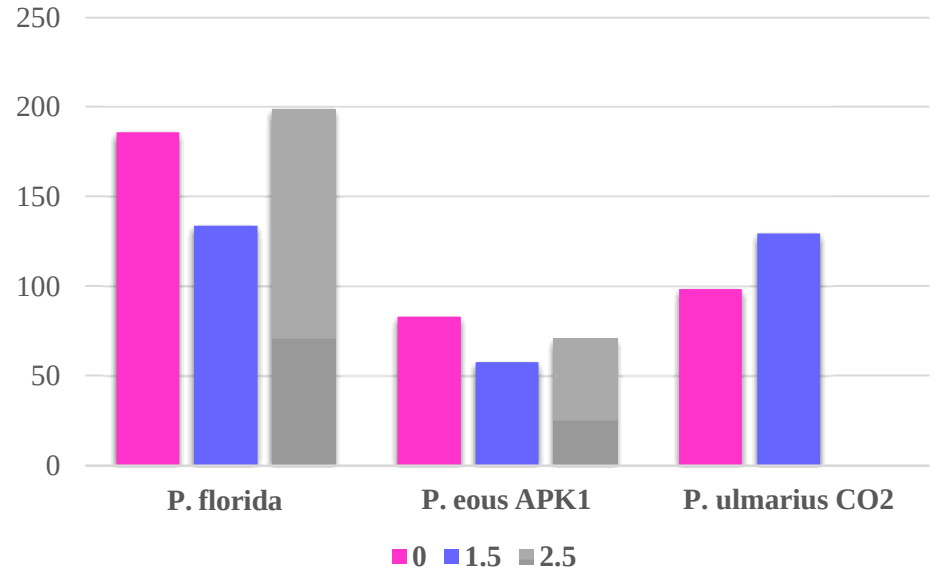
Effect of different concentrations of zinc enrichment on Flavonoid

Treatments	*P. florida*	*P. eous* APK1	*P. ulmarius* CO2
Control	185.67 ± 18.92	83.07±12.42	98.47±6.33
1.5 %	133.53 ± 52.08	57.47±9.16	129.40±47.82
2.5 %	198.60 ±31.55	70.58±8.63	
SEd **CD (P<0.05)**	**30.0603** **73.5583**	**8.3343** **20.3942**	**22.7399** **55.6451**

Values are expressed by mean ± SD of three samples in each group

Figure 12

Graphical comparison of flavonoids in the Pleurotus sp.



Zinc Enrichment

Zinc has biological significance for living organisms and mushrooms are known as good zinc accumulators (Isiloglu *et al*., 2001). Zinc content in the fruiting body was analysed to study the accumulation of the microelement in the fruiting body which was given in the form of Zinc Sulphate to enrich the mushroom.

Among the three *Pleurotus* Sp. the fruiting body of the *P.eous* APK1 strain was found to have accumulated the microelement when compared with *P. florida* and *P. ulmarius* CO2. P. eous APK1 was found to contain more zinc (37 mg / Kg) in the non-biofortified sample, while after biofortification the 1.5 % and 2.5 % of zinc sulphate enrichment resulted in 41.97 mg/ Kg and 49 mg/ Kg zinc respectively. In comparison with the other two *Pleurotus* Sp. it is evident that *P. eous* APK1 by itself has more zinc content and the zinc content has also increased. In *Pleurotus florida*, though there was an increase in zinc content, it has got decreased from 27.03 mg/ Kg to 17 mg / Kg respectively. *Pleurotus ulmarius* CO2 didn't show an increase in 1.5 % concentration, and a gradual increase of 24 mg/Kg from 23 mg/Kg was observed in 1.5 % and 2.5 % concentrations.

Zinc content was found to be 0.03 - 0.19 mg/100g for *Pleurotus ostreatus (EM-1) and Pleurotus eous (P-31)* respectively by Kortei and Kwagyan, 2015.

Antioxidant Activity

Antioxidant activity was found to increase in *P. eous* APK1 and *P. ulmarius* CO2 as the concentration of zinc sulphate bio-fortification increased, while it was found to decrease in *P. florida* and again there was a slight increase from 1.5 % to 2.5 % concentrations of Zinc sulphate biofortification of substrate (1.40 – 1.47) respectively.

Biofortification of the substrate with Zinc had a positive impact on the accumulation of zinc in the fruiting bodies except in *P. ulmarius* CO2. *P. ulmarius* CO2 showed very less uptake of zinc, whereas *P. florida* and *P. eous* APK1 showed an increase from control to 1.5 %. In the 2.5 % of zinc biofortification, in comparison to 1.5% of biofortification, there was a drastic decrease in *P. florida*, while *P. eous* APK1 showed an increasing trend in its accumulation from 27 – 17 and 42 – 49 respectively. When compared between the three species *P. ulmarius* growth was affected when others had an increase in the number of mushrooms produced. According to Gadd, (2007) some fungal mechanisms may have contributed to this tolerance, for example, a reduction

Table 18

Effect of different concentrations of zinc sulphate on zinc accumulation

Treatments	*P. florida*	*P. eous* APK1	*P. ulmarius* CO2
Control	21.0 ± 0.30	37.00±0.20	23.03±0.45
1.5 %	27.03 ± 0.45	41.97±0.15	23.00±0.20
2.5 %	17.0 ±0.40	49.00±0.40	24.00±0.40
SEd **CD (P<0.05)**	**0.3174** **0.7767**	**0.2228** **0.5451**	**57.6452** **141.0591**

Values are expressed by mean ± SD of three samples in each group

Figure 13

Graphical comparison of Zinc accumulation after zinc sulphate addition in the substrate in the three Pleurotus sp.

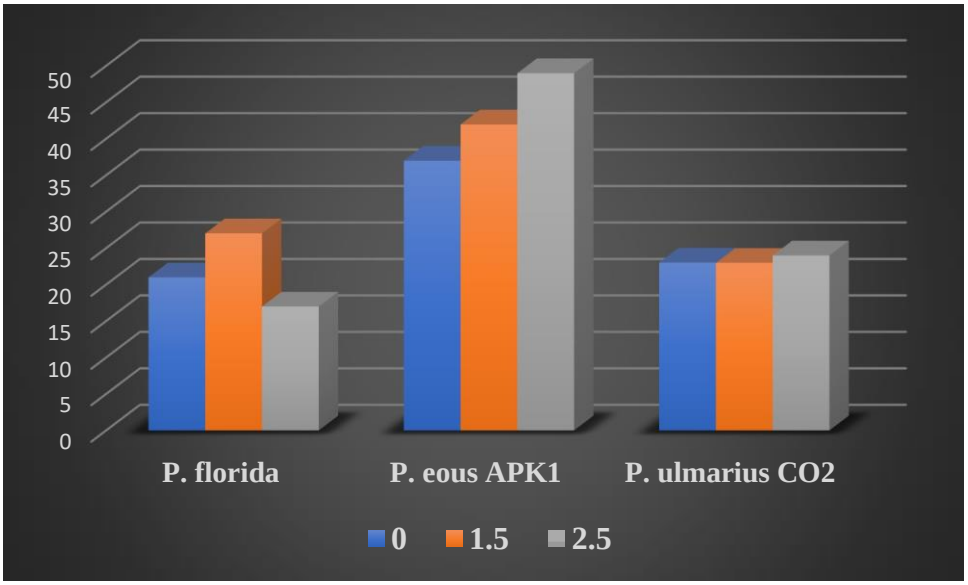
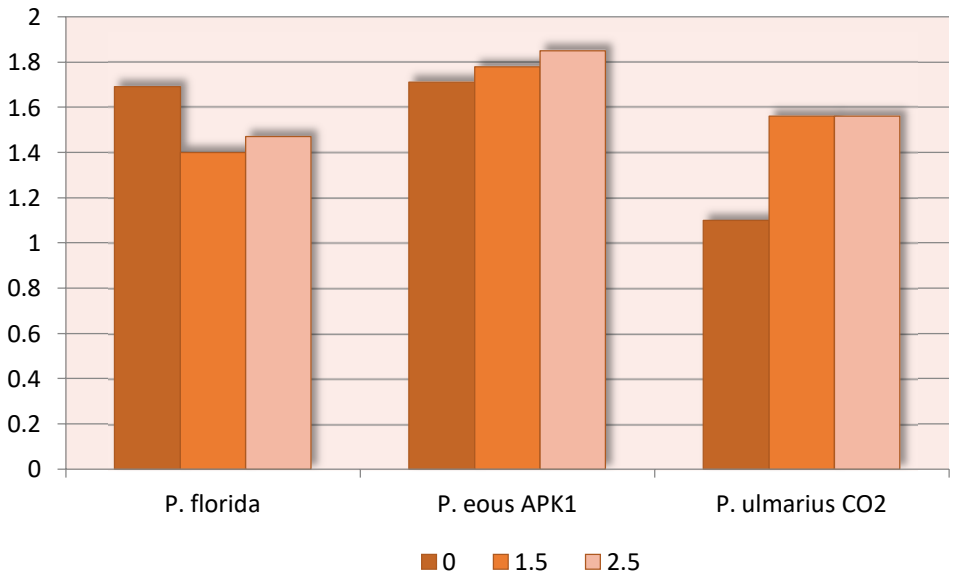


Table 19
Effect of different concentrations of zinc enrichment on AA%

Treatments	*P. florida*	*P. eous* APK1	*P. ulmarius* CO2
Control	1.69±0.08	1.71±0.11	1.10±0.44
1.5 %	1.40±0.13	1.78±0.06	1.56±0.05
2.5 %	1.47±0.23	1.85±0.04	1.56±0.10
SEd	**0.1471**	**0.0585**	**0.2169**
CD (P<0.05)	**0.4083**	**0.1623**	**0.6203**

Values are expressed by mean ± SD of three samples in each group

Fig 14 ***Graphical comparative study of effect on Antioxidant activity %***



of absorption or an increase in the efflux of metals through cell wall adsorption, the precipitation of minerals and polysaccharides, or extracellular binding by intracellular sequestration of metallothionein.

According to Gebrelibanos *et al*. (2016) zinc was found in all the analyzed fruiting bodies of the mushrooms with concentrations ranging from 89.68 ± 2.28 mg/kg in *Pleurotus ostreatus* and 95.26 ± 2.20 mg/kg in *Pleurotus florida*. The values for zinc in the investigated mushroom samples were above the permissible limit of 60 mg/kg recommended values in foods (WHO 1982). The average zinc content of the analyzed mushroom samples was 92.47 mg/kg. The values for zinc in the investigated mushroom samples were above the permissible limit of 60 mg/kg recommended values in foods (WHO 1982). Zinc concentrations of edible mushroom samples in the literature have been reported to be: 26.7 – 186.0 mg/kg (Genccelep *et al*., 2009), 19.0 – 49.7 mg/kg (George *et al*. 2014), 5.7 – 97.5 mg/kg (Sen *et al*., 2012), 29.0 – 146.0 mg/kg (Sarikurkcu *et al*., 2011), 27.8 – 70.9 mg/kg (Okwulehie & Ogoke 2013) and 6.4–27.33 mg/kg (Udochukwu *et al*., 2014). This reveals that the findings of this study are in good *P. pulmonarius* zinc content was 113.4 mg/ 100g while after Zn biofortification it was recorded as 349.5 mg/100 g (Oyetayo *et al*., 2021). Zinc present in dried *P. florida* was 16 mg/ 100g (Alam *et al*., 2018)

Summary and Conclusion

Summary and Conclusion

Mushrooms have a great ability to accumulate some elements that are essential for their metabolic functions, hence, supplementation of the substrate with different macro and micro-nutrients will efficaciously improve the nutritional status of cultivated fruiting bodies.

Zinc enrichment was done by the addition of zinc sulphate at two different concentrations (1.5 % and 2.5 %) to the paddy straw substrate. *Pleurotus florida, Pleurotus eous* APK1 and *Pleurotus ulmarius* CO2 are taken for the present study and the first harvest is done between 19-35 days. The growth of *P. ulmarius* CO2 at 2.5 % was affected and the yield was less in all the triplicates.

The biological efficiency among the non-biofortified mushrooms was calculated and found that *P. florida* (118.03) was higher than the other species taken for the study. But in *P. florida* the BE% was found to be decreasing as the concentration of zinc sulphate enrichment increased.

Ash content is high at 1.5 % biofortification of zinc. The moisture content is high in *P. florida*, after biofortification, the moisture content has been seen to get reduced and in *P. ulmarius* CO2 after biofortification, the moisture content was found to increase.

Phytochemical screening revealed the presence of Carbohydrates, Proteins, Vitamin C, alkaloids, Saponin, Steroids, Tannins, Phenolics and Flavonoids and the absence of Glycosides, Triterpenoids, and Anthroquinone in all the three species of *Pleurotus* with their different concentrations of Zinc sulphate applications.

Carbohydrate content was more in P. ulmarius when compared with the other two Pleurotus species. Biofortification with Zinc increases the carbohydrate content in *P.ulmarius.* Protein was also found to be higher in non-biofortified *P. florida* has a steady increase in zinc enrichment. From the above findings, it is been inferred that zinc biofortification increases carbohydrate and protein content in *P. florida.* Biofortification in *P. florida and P. eous* APK1 shows that there is an increase in the accumulation of fat and the total energy in the fruiting bodies than in the non-biofortified mushrooms.

Total phenolic content in *P. ulmarius* of non-biofortified mushrooms was found to be more when compared with *P. eous APK1* and *P. florida.* After enrichment *P. florida* and *P. eous* APK1

showed an increase in 1.5% concentration of zinc, while at 2.5 % concentration *P. florida* alone showed a drastic downfall in the phenolic content present in it. Tannin content was analysed where, tannin content was found to be less in *P. eous* APK1 and a decreasing trend was seen in all the biofortified *Pleurotus* species.

Vitamin C which shows the antioxidant capacity was also analysed and found that *P.eous* APK1 was having a higher amount of vitamin C than the other two species, but the zinc enrichment did not help in any increase at a bigger level. But *P, florida* which had a less amount of Vitamin C had a drastic increase, and also it was having more vitamin C than the other two species. This shows that with regards to Vitamin C content, biofortification has given a positive result.

Flavonoids were present more in *P. florida* in the non-biofortified sample, while after biofortification. Though there is a decrease in the level of flavonoids present in *P. florida* compared to the biofortification and non-biofortified mushrooms, it has high content when compared with other species.

Zinc accumulation is studied to ensure the uptake of zinc from zinc sulphate supplied to the substrate used for growing the three mushroom species. *P. florida* has not shown an increase at 1.5 % concentration of zinc sulphate, but at 2.5 % concentration, it has decreased. P. ulmarius has shown only a mere slight increase from the non-biofortified mushrooms, clearly stating that there is no uptake. Even though the growth was less and at 2.5 % concentration, the yield was very low in all the triplicates. But whereas in *P. eous* APK1 the non-biofortified sample itself had more zinc when compared to others, and also there is a steady increase in the accumulation of zinc from the supplied zinc sulphate. This states that the mycelium didn't have any negative impact on the growth.

Zinc there are many positive increases due to the zinc accumulation, we did antioxidant activity % to know the effect of biofortification Zn. AA % was increased in all the concentrations in *P.eous* APK1. In *P. florida* the AA % has got reduced in biofortified mushrooms.

Further investigations on the possible accumulations in various tissues and also the synergistic toxic effects will be the aspects of considerable attention.

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