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Intraspecific hybridization of *Pleurotus djamor* (Agaricomycetes) for developing short duration, high-yielding improved hybrid strain

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ABSTRACT

Pleurotus is a dominant genus comprising several edible species of commercial importance. In recent years, there has been a demand for developing improved strains with superior nutritional attributes. Breeding of improved strains for shorter duration, high biological efficiency that is, the total yield relative to the dry substrate weight, becomes essential to make hybrid strains economically more attractive compared to parents with improved nutritional and functional properties linked with health benefits is an emerging concept. The present experiment was conducted during 2021 and 2022 at Agricultural College and Research Institute (Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu), Madurai, Tamil Nadu to develop three intraspecific hybrids, viz. K2M2, K4M4 and K7M2 with blended characters of both parents by crossing between *Pleurotus djamor* isolate woody-1 and *P. djamor* var. MDU-1. Hybrid strains K2M2 and K4M4 were found promising and produced 1st pinhead in shorter duration of 12–15 days with a total duration of 35–40 days. Hybrid K2M2 produced maximum yield of 587.66 g/500 g of paddy straw substrate on a dry weight basis with biological efficiency of 117.53% followed by K4M4. In this study the developed hybrids were subjected to determine the bioactive secondary metabolites through Gas Chromatography-Mass Spectrometry (GC-MS) and proximate composition. Analysis of secondary metabolites revealed that hybrids and parents had specific secondary metabolites at differential levels. Also proximate analysis and organoleptic evaluation revealed that hybrids possessed improved nutritional and sensory characters compared to parents. Hybrid cultivars possesses good sensory traits, better yield and shorter duration than parents have been found to increase the number of cropping cycles annually along with improved nutritional and health benefits for the benefit of mushroom farmers.

Keywords: Biological efficiency, Early duration, Hyphal anastomosis, Intra-specific hybrids, Neutraceutical properties, *Pleurotus* spp.

In recent years, commercial mushroom production has increased worldwide contribution in food security and providing high economic gains for the country. More than 2,000 mushroom spp. exist in nature. Among the commercial mushrooms, the genus *Pleurotus* comprises a heterogeneous group of edible species and stands second in world production (Sharma *et al.* 2017). Mushrooms are a good source of non-starchy carbohydrates, proteins, vitamins, minerals and contain certain bioactive compounds responsible for anti-oxidant, antimicrobial and antitumor properties (Rosli *et al.* 2015). Mushrooms have been reported to be of therapeutic value, useful in managing certain diseases such as hypercholesterolemia, hypertension and cancer, and also having antiviral and antibacterial properties. In addition to high nutritional and therapeutic properties, *Pleurotus* spp. have high biological efficiency, as they are efficient cellulose and lignin degraders and able to grow on varied agricultural substrates by degrading both cellulose and lignin which in turn produce fruiting bodies. Mushrooms are also rich in vitamins and minerals and contain an abundance of essential amino acids. Therefore, mushrooms are good supplement to cereal grains (Sadler 2003).

Generally, the productivity and quality of edible mushrooms depend mainly on the genetic constitution of the selected strain (Kaur and Sodhi 2012). Strain improvement is usually achievable through selection and hybridization. The objective of the mushroom breeding programme is to bring together superior traits from two parents that lead to the selection and creation of superior desired traits (Miles 2018). Somatic hybridization between *Pleurotus* spp. is

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an emerging concept in developing hybrid strains with desirable traits. In this regard, developing elite strains, with desired traits such as shorter duration and higher yield with good palatability and improved nutraceutical properties, is the need of the hour. Thus, this study aims to develop hybrids by crossing *P. djamor* isolate woody-1, having short cropping duration, early flushing with higher yield but poor palatability, with *P. djamor* var MDU-1, having good palatability but long cropping duration, to get hybrid strain having short cropping duration, good palatability and high yielding potential along with improved nutritional and secondary metabolite production.

MATERIALS AND METHODS

Source of parent cultures and growth conditions: An experiment was conducted during 2021 and 2022 at Agricultural College and Research Institute (Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu), Madurai, Tamil Nadu. The experiment was laid out in four trials, viz. January–June 2021, July–December 2021, January–June 2022 and July–December 2022. The mycelial cultures of the mushroom spp. used for the hybridization process, viz. *P. djamor* isolate woody-1 and *P. djamor* var. MDU-1 were obtained from Agricultural College and Research Institute (Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu), Madurai, Tamil Nadu. They were cultured on Potato Dextrose Agar (PDA) medium at 28°C and grown-up cultures were maintained at 4°C.

Spawn and mushroom bed preparation: The pure cultures of *P. djamor* woody-1 and *P. djamor* MDU-1 were inoculated in the sterilized sorghum grain as a substrate for spawn preparation (Krishnamoorthy *et al*. 2015). The inoculated bags were incubated in a spawn room at 20±2°C. Days to complete colonization of mycelium on the sorghum grain (spawn) substrates were recorded. Fully mycelia-covered sorghum grains were used as spawns for mushroom cultivation using paddy straw as a substrate (mushroom bed preparation). The inoculated mushroom beds were incubated in the mushroom shed at 25°C and RH >80% for initiation of basidiocarps. The basidiocarps were harvested two days after pin head initiation. The mean yield after three harvests in four trials was recorded, and biological efficiency was estimated for the parents *P. djamor* woody-1 and *P. djamor* MDU-1.

Intraspecific hybridization between P. djamor *woody-1 and* P. djamor *MDU-1*: *P. djamor* isolate woody-1 and *P. djamor* var MDU-1 were used as the parents for intraspecific hybridization. The parent *P. djamor* isolate woody-1 is an early maturing, having a short cropping duration of 30 days but it is less palatable having less plectenchyma tissue, rough texture and feels leathery while chewing. It was crossed with commercially available delicate, high yielding *P. djamor* var MDU-1 were highly palatable with good yield but a long duration of 45–50 days. Freshly harvested matured basidiocarps of parents *P. djamor* isolate woody-1 and *P. djamor* var MDU-1 were used for the collection of basidiospores. The basidiospores were collected by spore print method (Petersen and Ridley 1996).

The shredded basidiospores were collected by adding 10 ml of sterilized water (basidiospore stock suspension). The basidiospore stock suspension was prepared for each parent and was serially diluted and inoculated in the PDA plates by spread plate technique to get spore concentration of 30–100 basidiospores/plate were incubated at 28°C for 4–6 days or until small individual white mycelial colonies appear on the petri plates with the diameter of 3–5 mm (Bahukhandi and Sharma 2002). Fast-growing monokaryotic colonies having typical radial growth were selected phenotypically from spread plates of *P. djamor* woody-1 and *P. djamor* MDU-1 and subcultured on fresh PDA plates. Ten monokaryons, confirmed based on the absence of clamp connections, were selected from each parent for hybridization.

Intraspecific hybridization was carried out between *P. djamor* isolate woody-1 and *P. djamor* var MDU-1 through a dual culture plating technique (Kumara and Edirimanna 2009). The formation of compatible mating pairs was phenotypically selected by prominent interaction between parents and the appearance of bright fluffy putative, thick white coloured vigorous mycelial growth at the contact zone of two monokaryotic mycelia which was confirmed by the formation of clamp connection in the mycelium under microscope. This dikaryotic mycelium or hybrid mycelia, was cut from the contact or merged region and sub-cultured on the fresh PDA plates and incubated at 28°C for 5 days. Whereas the formation of a barrage region or lytic zone at the merger point between two parents was found to incompatible mating pairs. Spawn and bed preparation were undergone from the dikaryotic mycelium. The dikaryotic mycelia thus obtained were screened for basidiocarp formation and proximate composition.

The mean yield in four trials and biological efficiency was estimated for the developed intraspecific hybrids, which were compared with their parents. Finally, 3 hybrids, viz. K2M2, K4M4 and K7M2 having short duration, early flushing with high biological efficiency were obtained. The obtained hybrids (K2M2, K4M4 and K7M2) and their parents (*P. djamor* woody-1 and *P. djamor* MDU-1) were subjected to proximate analysis and secondary metabolite production.

Nutritional analysis of parents and hybrid mushroom strains: The parent and hybrid mushroom samples were harvested at the matured stage of two days of pin head initiation. Dried mushroom samples were subjected to proximate composition, viz. protein AOAC (1995), crude fibre, crude fat and total ash (James 1995). The carbohydrate and total energy value (Alam *et al.* 2008) were estimated following standard procedures.

Identification of secondary metabolites by GC-MS: The freeze-dried mushroom samples (1 g) of parents and hybrids were mixed with 40 ml of methanol solvent and ultrasonicated for 15–30 min. The obtained extract was filtered and was concentrated in the rotary evaporator at 50°C and 150 rpm for 10 min. The crude concentrate was diluted with 2 ml of methanol and filtered through Millipore

filter of 0.22 µ pore size, and the clear crude solution was collected. Then the prepared samples were subjected to GC-MS analysis. GC-MS analysis of parental and hybrid oyster mushroom samples with methanol extract was analyzed by using Shimadzu GC-MS QP- 2020 system. The relative percentage amount of each component present in hybrid strains and their parents was calculated by comparing its average peak area to the total areas (Keshamma 2022).

Statistical analysis: Analysis of variance was carried out to find the differences between each treatment and Duncan's Multiple Range Test were analysed for comparison of mean by using SPSS 16.0 software

RESULTS AND DISCUSSION

Assessing the cropping period and yield performance of parents and hybrid strains: In this study, 10 fast-growing monokaryons of each parental strain, *P. djamor* woody-1 (K1–K10) and *P. djamor* MDU-1 (M1–M10) were selected phenotypically. The selected monokaryons were crossed at all possible combinations as a dual culture resulting in 100 crosses. Among the hundred crosses made, 21 crosses appeared to be compatible pair phenotypically. The fluffy growth at the merger region depicted the putative dikaryon formation. Whereas the remaining pairs were imperfect in growth and found incompatible by the formation of a lytic zone at the junction point or merger region of two monokaryotic mycelia. Among the 21 compatible dikaryotic mycelia, three mating pairs/hybrids, viz. K2M2, K4M4 and K7M2 were able to produce basidiocarp during mushroom cultivation.

Comparing the parents and the obtained hybrids for pinhead initiation, the hybrids K2M2 and K4M4 exhibited desirable traits of early basidiocarp formation, where it produced 1st pin head in minimum duration of 13.00 and 14.33 days, respectively. Even though, the number of days of pin head initiation of hybrids was slightly higher than the wild type parent *P. djamor* woody-1 (10.33 days) it was lesser compared to the commercially cultivating parent *P. djamor* var. MDU-1 (22.33 days) (Table 1). The results were in accordance with Reihana *et al.* (2018), who observed that *P. djamor* strain woody-1 possessed the shortest period for pinhead formation due to faster substrate colonization with the shortest duration of 29–30 days. Similarly, days required for pinhead initiation were significantly reduced in hybrids by two and three days compared to parent (Jyothi and Thara 2021). Likewise, Sindhu *et al.* (2022), reported that interspecific hybridization between *P. djamor* woody-1 and *P. djamor* MDU-1 led to produced hybrids with primordial initiation in 9–10 days after bed preparation. Likewise, total cropping duration for the *P. djamor* woody-1 and hybrids, viz. K2M2 and K4M4 ranged between 30 to 35 days and for hybrid K7M2 and the other parent *P. djamor* var. MDU-1 required 42.00 and 46.00 days respectively to complete total cropping period (Table 1). Thus, this study indicated that among three hybrids tested for the study, hybrid strains K2M2 followed by K4M4 were found to have a short duration compared to parent *P. djamor* var MDU-1.

The hybrid K2M2 recorded the highest yield of 587.66 g with biological efficiency (BE) of 117.53% followed by *P. djamor* woody-1 (578.33 g, 115.66% BE), hybrid K4M4 (546.33 g, 109.26% BE). Whereas, the least yield was observed in hybrid K7M2 (513.33 g, 102.66% BE) which is at par with parent *P. djamor* var. MDU-1 (516.83 g, 103.66% BE) (Table 1, Fig. 1). As the result of intraspecific hybridization, hybrids K2M2 followed by K4M4 possessed the mixed traits of shorter duration from parent *P. djamor* isolate woody-1 and higher yielding trait from commercially cultivating parent *P. djamor* var. MDU-1. In addition to this, phenotypic dominance of *P. djamor* woody-1 has been observed over *P. djamor* var MDU-1 in all the hybrids obtained. This could be achieved by the segregation of genes controlling these desirable traits into these two hybrids. In addition, both hybrids showed a heterosis effect on biological efficiency, giving higher yields than their parents. The results were in accordance with the study of Avin *et al.* (2016) who described the development of intraspecific hybrid oyster strains of *P. pulmonarius* possessed short cropping duration and high yielding capacity compared to their parent's strains. Similarly, hybridization was made between *Pleurotus* spp., resulting in a production of two hybrids having better texture, less odour and the higher yield compared with their parental strains (Rosnina *et al.* 2016). Likewise, Ogunleye *et al.* (2020) and Jyothi and Thara (2021) observed that the average flush yields were higher in developed new strains with increase in basidiocarp weight compared to parents. The above mentioned studies,

Table 1 Pooled data analysis of days needed for pinhead appearance and total duration of parents and hybrids

Pleurotus strains	Days required for pin head appearance	Days required for harvest			Yield (g/500 g)	BE (%)
		1st flush	2nd flush	3rd flush		
P. djamor woody-1	10.33^{c}	12.66^{e}	21.00^{d}	30.33^{d}	578.33^{a}	115.66
P. djamor MDU-1	22.33^{a}	24.66^{a}	36.00^{a}	46.00^{a}	516.83^{c}	103.36
K2M2	13.00^{b}	15.00^{d}	24.66^{c}	33.00^{c}	587.66^{a}	117.53
K4M4	14.33^{b}	16.66^{c}	25.33^{c}	35.00^{c}	546.33^{b}	109.26
K7M2	21.00^{a}	23.33^{b}	31.66^{b}	42.00^{b}	513.33^{c}	102.66
CD (P=0.05)	2.15	1.24	2.69	2.04	16.27	
CV	8.04	4.07	6.42	3.35	1.62	

*Mean of four trials each with three replications. The treatments were compared by Duncan's Multiple Range Test (DMRT) analysis. BE, Biological efficiency.

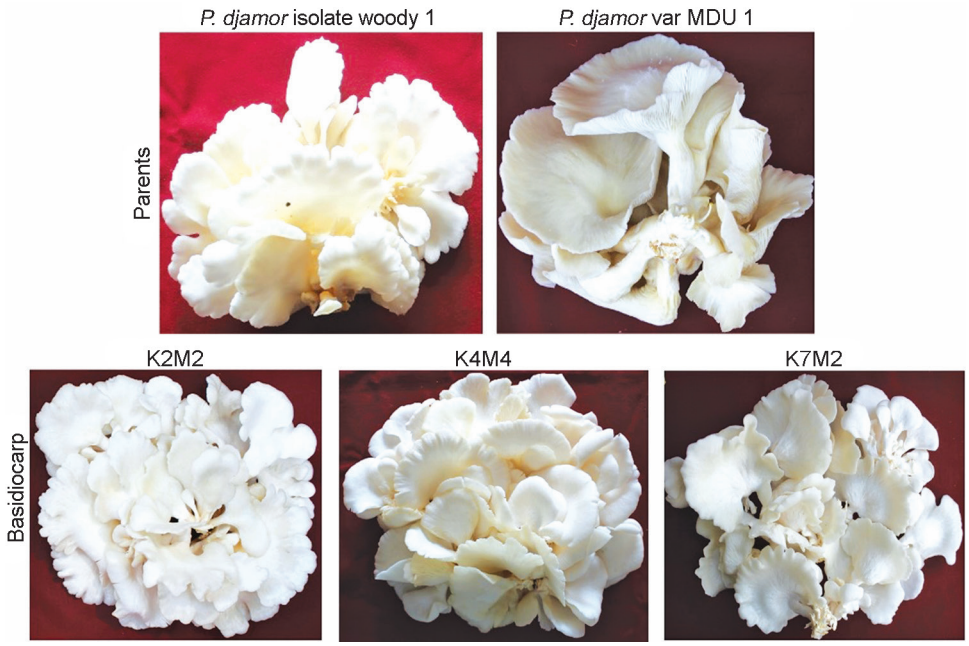

Fig. 1 Morphology of basidiocarps of parents and hybrids of *Pleurotus* spp.

along with our experiment, indicated that the hybrid strain in oyster mushrooms of desired traits viz. short cropping duration with improved yield, can be developed through intraspecific hybridization.

Proximate composition of hybrid and parental oyster mushroom: The chemical and proximate composition of edible mushrooms determines their nutritional value and sensory properties. Significant variation was noticed in protein, crude fiber, fat, carbohydrate and energy value of parents and hybrid mushrooms (Table 2). In this study, protein content of parents and hybrid mushroom spp. ranged from 18.66–26%. The parent *P. djamor* MDU-1 and the hybrid K2M2 possessed higher protein content of 26.00%. Whereas the parent *P. djamor* woody-1 contains a minimum protein percentage (18.66%).

The percentage of crude fiber in parents and hybrid mushrooms ranged from 11–16.66%. The hybrids K2M2 and K4M4 possessed 14.00 and 13.66% crude fiber which are intermediaries to their parents. Higher fat content was observed in parent *P. djamor* woody-1 with 2.16%. In contrast, the hybrid K2M2 possessed minimum fat content of 0.60%. Similarly, higher carbohydrate (56.20%) and energy value (336.00 Kcal 100/g) were noticed in *P. djamor* MDU-1 which was closely followed by hybrid strain K2M2 with carbohydrate and energy value of 55.07% and 329.68 Kcal 100/g, respectively. Whereas the least carbohydrate (52.86%) and energy value (305.52 Kcal 100/g) was observed in another parent *P. djamor* woody-1 (Table 2). Several workers reported that high protein with low fat are the primary characteristics for mushroom (Alam *et al.* 2008). These are in line with the present study the hybrids K2M2 followed by K4M4 possessed higher protein with minimum fat content compared to parents. The results of the present study are in accordance with that of Selvakumar *et al.* (2015) and Jyothi and Thara (2021) who reported that the hybrids were superior to their parents in nutritional aspects than their parental strains.

Sensory evaluation of parents and hybrids revealed that *P. djamor* var MDU-1 was the most preferred strain with overall acceptability of 8.50 which were followed and statistically similar with hybrid K2M2 as it gained acceptability of 7.60 score followed by K4M4 (7.50). Whereas, *P. djamor* isolate woody-1 recorded the lowest mean score value for overall acceptability (6.13) and felt leathery and slightly hard while eating and chewing.

Analysis of secondary metabolites by GC-MS: Through GC-MS analysis, a total of 50 secondary metabolites were identified in parents and hybrids. Hybrids and parental strains exhibited significant variations in the relative proportion of secondary metabolites. Among the different metabolites obtained, fourteen compounds were present at higher levels in hybrid strains than their parents (Table 3). The heat map

Table 2 Pooled analysis of proximate constituents parents and hybrid strains

Pleurotus strains	Protein (%)	Crude fibre (%)	Fat (%)	Carbohydrate (%)	Energy value
P. djamor woody-1	18.66 ± 1.15^{c}	16.66 ± 0.57^{a}	2.16 ± 0.05^{a}	52.86	305.52
P. djamor MDU-1	26.00 ± 1.00^{a}	11.00 ± 1.00^{c}	0.80 ± 0.10^{c}	56.20	336.00
K2M2	26.00 ± 1.00^{a}	14.00 ± 1.00^{b}	0.60 ± 0.10^{d}	55.07	329.68
K4M4	24.00 ± 1.00^{b}	13.66 ± 1.15^{b}	0.83 ± 0.05^{c}	54.51	321.51
K7M2	23.66 ± 1.15^{b}	11.33 ± 1.52^{c}	1.43 ± 0.11^{b}	54.92	327.19
CD (*P*=0.05)	1.93	1.99	0.16		
CV	4.49	8.21	7.66		

*Mean of four trials each with three replications. Values were given in Mean ± Standard deviations. The treatments were compared by DMRT analysis.

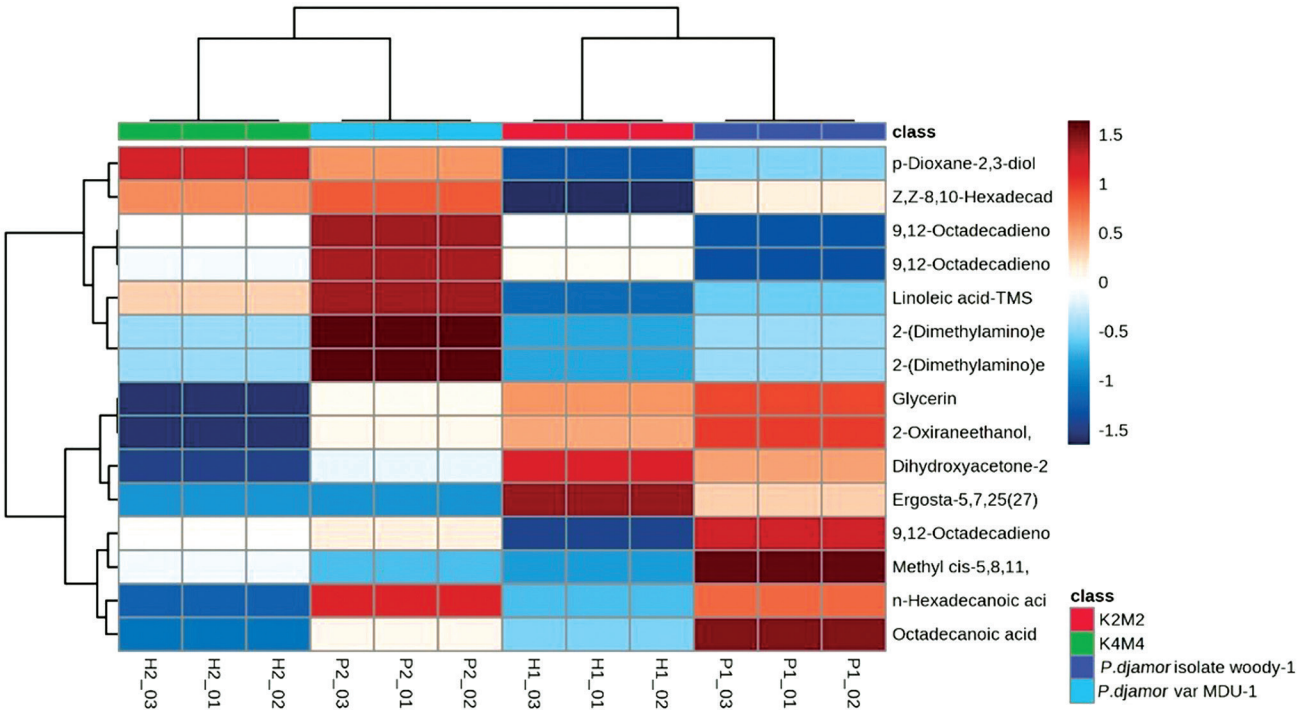

Fig. 2 Heat map for comparing secondary metabolites present in parent and hybrid strains.

Table 3 List of secondary metabolites found in parents and hybrid *Pleurotus* strains and their therapeutic usages

Compound	Area (%)				Therapeutic use	References
	P. djamor woody-1	*P. djamor* MDU-1	K2M2	K4M4		
9,12-Octadecadienoic acid (Z,Z)	1.43	65.69	41.45	68.84	Diuretic, swelling and detoxification properties	Peng *et al.* (2013)
n-Hexadecanoic acid (Palmitic acid)	9.66	8.95	10.36	10.48	Anti-inflammatory	Aparna *et al.* (2012)
Octadecanoic acid	3.21	0.85	3.30	3.87	Antibacterial	Younis *et al.* (2019)
9,12-Octadecadienoic acid (oleic acid)	0.10	3.00	2.84	3.72	Boost memory and reduce blood pressure, antibacterial	Younis *et al.* (2019)
Dihydroxyacetone	0.22	0	2.66	0	Antimicrobial	Younis *et al.* (2019)
Glycerin	0.98	0	2.39	0	Antioxidant properties	Aparna *et al.* (2012)
2-Oxiraneethanol	1.01	0	2.04	0	Antimutagenic	Aparna *et al.* (2012)
Ergosta-5,7,25 (27)-trienol	0.10	0	1.30	0.20	antioxidant, anticancer, antidiabetic, anti-inflammatory, hepatoprotective, antiallergic, antimicrobial activities precursor of vitamin D,	Jiji and Subin (2017)
2-(Dimethylamino) ethyl vaccenoate	0.32	1.16	1.00	2.19	Antibacterial	Peng *et al.* (2013)
p-Dioxane-2,3-diol	0	0.10	0	3.17	Anticancer, pancreaprotective and antiasthmatic activity	Jiji and Subin (2017)
9,12-Octadecadienoic acid,dihydroxypropyl ester	1.43	0	0	2.36	Antioxidant	Jiji and Subin (2017)
Z,Z-8,10-Hexadecadien-1-ol	0.10	0.22	0	1.50	Antifungal, Antibacterial, Nematicidal	Kim *et al.* (2020)
Linoleic acid-TMS	0.10	0.20	0.30	1.10	Antioxidant anti-inflammatory, omega 6- fatty acid, essential fatty acid good for heart	Kim *et al.* (2020)
Methyl cis-5,8,11,14,17-Eicosapentaenoate	0.92	0..20	0.32	1.10	Anti-inflammatory, good for health	Peng *et al.* (2013)

was constructed from the above-obtained metabolites using Metaboanalyst 5.0 software (Fig. 2).

Among 14 compounds obtained, 7 compounds were found in higher level in hybrid K2M2 compared to parents. Among them, n-Hexadecanoic acid (Palmitic acid) (10.36%) found in higher level in K2M2 followed by Octadecanoic acid (3.30%) (Table 3). Whereas, metabolite compound, 9, 12-Octadecadienoic acid (Z,Z)- found higher level of 68.84% in hybrid K4M4 as compared to parents followed by 9,12-Octadecadienoic acid and methyl ester (oleic acid) (3.72%). The therapeutic use of metabolites in human health benefits were mentioned in Table 3. Higher levels of secondary metabolites in hybrid strains indicated the successful hybridization of both parents. The presence of volatile metabolites in specific percentage is due to the genetic makeup of the strain along with environmental factors, especially the substrates used for mushroom cultivation (Peng *et al*. 2013, Tagkouli *et al.* 2021) which is in accordance with the current study that a higher amount of secondary metabolites presents in hybrid strain compared to their parents could be due to the genetic makeup of the hybrid and parental strains.

From this study, the hybrids K2M2 followed by K4M4, possessed the desired attributes, namely, quick initiation of pinheads in a short period, short duration and high yield/biological efficiency compared to parent *P. djamor* MDU-1, a commercially cultivated ruling oyster variety in Tamil Nadu. Proximate composition revealed that the hybrids K2M2 and K4M4 had better protein, crude fiber with lower fat and carbohydrate percent than their parents. It was also noted that both hybrids had higher amounts of certain secondary metabolites than their parents. In addition, the hybrid had improved sensory attributes than *P. djamor* woody-1, another parental strain. Hence, hybrids K2M2 and K4M4 could be suggested for commercial cultivation and utilized for the benefit of mushroom farmers. However, a detailed study needs to be conducted to obtain reproductive results and an improved amount of vitamins, minerals and specific antioxidants present in hybrid mushrooms which will attract more consumer preference for hybrids because of improved health benefits.

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