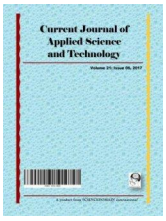

Current Journal of Applied Science and Technology

26(5): 1-10, 2018; Article no.CJAST.40423
ISSN: 2457-1024
(Past name: British Journal of Applied Science & Technology, Past ISSN: 2231-0843, NLM ID: 101664541)

Selection of Culture Media and Monokaryotic Isolates of *Pleurotus flabellatus* and *P. sajor-caju* for their Dikaryotization Followed by Performance Testing of Dikaryotic Isolates on Malt Extract Agar Medium

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Authors' contributions

This work was carried out in collaboration between all authors. Author DB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AR designed the study. Authors ST and DC managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CJAST/2018/40423
Editor(s):
(1) A. A. Hanafi-Bojd, Assistant Professor, Department of Medical Entomology & Vector Control, School of Public Health, Tehran University of Medical Sciences, Iran.
Reviewers:
(1) R. Mahalakshmi, India.
(2) Fatih Kalyoncu, Manisa Celal Bayar University, Turkey.
Complete Peer review History: http://www.sciencedomain.org/review-history/23933

Original Research Article

Received 28th February 2018
Accepted 31st March 2018
Published 3rd April 2018

ABSTRACT

Aims: The study was to find out the suitable agar medium and the selection of monokaryotic islates of *P. flabellatus* and *P. sajor-caju* for its dikaryotization with the help of rapidity in the growth performance.
Place and Duration of Study: Department of Plant Pathology Uttar Banga Krishi Viswavidyalaya, West Bengal, India during the academic year of 2014-2016.
Methodology: Fifteen and Five monokaryotic isolates of *Pleurotus flabellatus* (PFm) and *P. sajor-caju* (PSCm) were grown on five different culture media viz., Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA), Oat Meal Agar (OMA), Malt Extract Agar (MEA), V^8 Juice Agar (V^8JA) for testing

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their rapidity in radial growth. After the selection of culture medium and monokaryotic isolates growth performance was again conducted for the selection of dikaryotic culture on MEA.
Results: MEA was found most suitable for rapid radial growth of monokaryotic isolates *viz.,* PF9m, PF10m, PF11m, PF1m and PSC1m and was finally selected for dikaryotization (hybridization). Highest growth on MEA was observed in interspecific hybrids of PF×PS7 (16.40 mm/day) followed by PF×PS17 (16.29 mm/day), PF×PS19 (16.18 mm/day) and PF×PS13 (16.01 mm/day) respectively. Intra specific hybrids of PF×PF27, PF×PF10, PF×PF33, PF×PF24, PF×PF5, PF×PF25, PF×PF4, PF×PF12, PF×PF1, PF×PF26 and PF×PF13 shows the highest growth rate above 16 mm/day.
Conclusion: Dikaryotic mycelium showed the highest growth rate of above 16 mm/day as compare to monokaryotic isolates of *P. flabellatus and P. sajor-caju*.

Keywords: Pleurotus flabellatus; Pleurotus sajor-caju; monokaryotic; dikaryotization; Malt extract Agar.

1. INTRODUCTION

Mushrooms have been part of our human diet since time immemorial. They were used as food even before man understood the use of other microorganisms. Undoubtedly mushrooms were one of man's earliest foods which belong to the Kingdom Fungi due to unique fungal characteristics which draw a clear line from animals and plants. Unlike green plants, mushrooms are heterotrophs. Not having chlorophyll, they cannot generate nutrients by photosynthesis, but take nutrients from outer sources. They were often considered an exotic and luxurious food reserved for the rich. Today mushroom are food for both the rich and the poor. They can be grown anywhere as long as the conditions for their growth and cultivation are provided. Available mushroom technologies brought forward by making the new combination of genes into one individual through dikaryotization with the objective of higher yield and better nutritional properties. Oyster mushrooms (*Pleurotus spp.*) are most suited in country like India which is rich of agricultural wastes such as straw, saw dust; sugarcane bagasse and others are available. While looking into the present scenario of India, increase in air pollution by crop residue fires form a dense canopy of smoke in the month of October to November. Cultivation of mushroom is the most promising alternatives to utilize agricultural waste into a valuable product. Moreover, composting of *Pleurotus* is very easy and cheaper as compare to other mushrooms.

2. MATERIALS AND METHODS

2.1 Place of Experimentation

The laboratory experiments were done in Research Laboratory, Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya, Pundibari, Coochbehar, West Bengal, India.

2.2 Media

The culture media were sterilized in an autoclave at15 p.s.i. for 15 minutes. Followings were the media used during the investigation. 1) Potato Dextrose Agar [1], 2) Czapek Dox Agar [2], 3) Oat meal agar [3], 4) Malt extract agar, 5) V^8 Juice agar.

2.3 Mushroom Species Used and Their Maintenance

Fruit bodies from two *Pleurotus* species namely *P. flabellatus* and *P sajor-caju* were collected from KVK farm of Uttar Banga Krishi Viswavidyalaya. Pure cultures were made from the stipe of fruit bodies by tissue culture technique. From the tip of hyphal growth mycelial segment was taken and transferred to PDA slants to get the pure culture of individual species. The mother cultures were kept at 4°C for further use.

2.4 Isolation of Monosporus Cultures of *P. flabellatus* and *P. sajor-caju*

Isolation of monosporus culture is one of the most important steps to develop homokaryons before aiming of assembling new genes by dikaryotization in mushroom. Following two methods were followed to get the monosporus cultures of *P. fabellatus* and *P. sajor-caju*.

i. **Spore print method:** proposed by Petersen and Ridly [4] method was followed for the single spore isolation of both *P. fabellatus* and *P. sajor-caju* (Plate 1 & 2).

Plate 1. Discharging of spore from fruit body

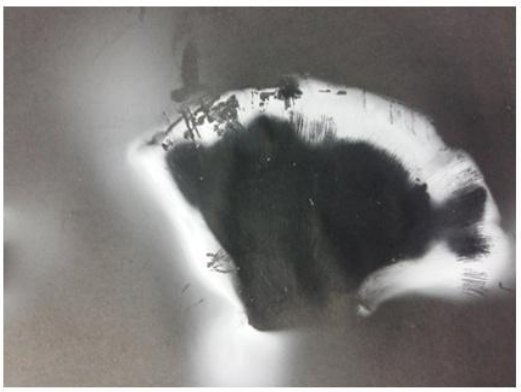


Plate 2. Spore print from fruit body

ii. **Serial dilution method:** as mentioned by Bahukandi and Sharma [5] was followed with little modification for the isolation of single spore. The fresh mushroom cap from *P. fabellatus* /*P. sajor-caju* was cut and dipped in sterilized water in Petriplate by putting gills at downward position for an hour (Plate 3). Afterwards that the cap was removed and suspension was further diluted up to 10^{-4}. The spore concentration having as low as 4-5 spores in lower power microscope of (10x) was selected. A sterilized loop was dipped in spore suspension and streaked on sterilized PDA in Petri plates. After incubation for 3 days, single discrete colonies were observed to develop, isolated and transferred in PDA slant.

2.5 Effect of Different Growth Media on the Performance of Monokaryotic Mycelium of *P. flabellatus* and *P. sajor-caju*

To find out the suitable medium for the growth of different monokaryotic isolates of *P. flabellatus* and *P. sajor-caju,* five different media *viz.,* potato dextrose agar media, Czapek-dox agar media, oat meal agar media, V^8 juice agar media and malt extract agar media were evaluated in 90 mm sterilized Petri plates each containing 15 ml of medium (Plate 7&8). *P. flabellatus* and *P. sajor-caju* isolates each showing higher average growth rate were selected for the dikaryotization.

2.6 Inter and Intra Specific Dikaryotization

Both inter and intra species dikaryotizations of isolates finally considered were done in Petriplates containing malt extract medium following dual culture plate techniques or Crossing between two monokaryotic *P. flabellatus* and or *P. sajor-caju* (Table 1 & 2) isolates was performed by taking mycelial disc of 5 mm diameter from periphery of seven days old monokaryotic cultures and placed in the two opposite sides of the Petriplates containing malt extract agar media (Plate 9 & 10). Thereafter, it was incubated for 3 days at 25±1°C. Small portion of dikaryotic hypha was taken by chopping from the meeting points of two different isolates. Dikaryotization was confirmed by the observing hyphal bridge or clamp connection (Plate 4 & 11). The inocula showing the dikaryotization were transferred to PDA slants and maintained at 4±C for further use.

Table 1. Parentage and interspecific hybrids of *Pleurotus*

Parentage	Hybrids
PF9m × PSC5m	PF× PS1, PF×PS2, PF×PS3
PF9m × PSC1m	PF×PS4, PF×PS5
PF10m × PSC5m	PF×PS6, PF×PS7, PF×PS8
PF10m × PSC1m	PF×PS9, PF×PS10
PF11m × PSC5m	PF×PS11, PF×PS12, PF×PS13
PF11m × PSC1m	PF×PS14, PF×PS15
PF1m × PSC5m	PF×PS16, PF×PS17m, PF×PS18
PF1m × PSC1m	PF×PS19, PF×PS20.

PF: Pleurotus flabellatus; PSC: P. sajor-caju

2.7 *In vitro* Selection of Potential Inter and Intra Specific Strains of *P. flabellatus* and *P. sajor-caju*

To screen out the fastest growing hybrid crosses the growth test was carried out in the 90 mm Petriplates containing 15 ml malt extract agar medium. Hybrid strains comprising of 20 inter species and 34 intra species of *P. flabellatus* and

P. sajor-caju was inoculated aseptically by 5 mm mycelial disc in a petripates having malt extract media and growth performance was recorded separately (Plate 12 & 13, Fig. 2 & 3). Further selection was made on the basis of growth performance and the potential of spawn production in the rice and wheat grain. Twenty inter and intra species was selected for further studies.

Table 2. Parentage and interspecific hybrids of *P. flabellatus*

Parentage	Hybrids
PFm9 × PF10m	PF×PF1, PF×PF2, PF×PF3,
PF9m × PF11m	PF×PF4, PF×PF5
PF9 m × PF1m	PF×PF6, PF×PF7, PF×PF8,
PF10m × PF9m	PF×PF9, PF×PF10, PF×PF11,
PF10m × PF11m	PF×PF12, PF×PF13, PF×PF14
PF10m × PF1m	PF×PF15, PF×PF16, PF×PF17,
PF11m × PF9m	PF×PF18, PF×PF19, PF×PF20,
PF11m × PF10m	PF×PF21, PF×PF22, PF×PF23,
PF11m × PF10m	PF×PF24, PF×PF25, PF×PF26
PF1m × PF9m	PF×PF27, PF×PF28, PF×PF29,
PF1m ×PF10m	PF×PF30, PF×PF31,
PF1m × PF11	PF×PF32, PF×PF33, PF×PF34,

The data obtained was subjected to statistical analysis using SAS and JMP software.

3. RESULTS AND DISCUSSION

3.1 Selection of Culture Medium for Growth of Monokaryotic Isolates of *P. flabellatus* and *P. sajor-caju*

Fifteen monosporus isolates of *P. flabellatus* designated as PF1m to PF15m and five strains of *P. sajor-caju* designated as PSC1m to PSC5m were grown in five different media *viz.,* PDA, CDA, V^{8}JA, MEA, OMA.

The results revealed that PDA followed by MEA was the most suitable for their growth and no significant difference was observed between them. Irrespective of strains, the mean mycelial growth rate between 7th-9th day of inoculation on PDA and MEA were 13.51 mm/day and 13.31 mm/day, respectively. In PDA the mycelial growth rate from ranged from 8.98-17.15 mm/day, PF9m showed maximum growth rate (17.15 mm/day) followed by PF10 (16.48 mm/day) whereas PF13 exhibited the lowest. In MEA highest growth rate was found in PF11m (15.72 mm/day) followed by PF4m (15.52 mm/day). In V^{8}JA and OMA Medium the growth rate ranged between 5.13-12.50 mm/day and maximum was with isolate PF8 whereas in OMA medium growth rate ranged from 6.81-13.00 mm/day and maximum was observed in PF10m. Lowest growth was observed in CDA, where the growth rate was ranged between 3.06-9.48 mm/day. From the overall growth performance of the monokaryons of *P. flabellatus*, four strains namely PF9m, PF10m, PF11m and PF1m were selected as parents to be used in interspecific hybridization (Table 3). It was also to be noted that media had significant effect on growth of monokaryotic strains of *P. flabellatus* (Fig. 1) and the strains also differed significantly irrespective of media tested (Fig. 2).

Similar effect was also observed in growth performance of monokaryons of *P. sajor caju*. The strains differed significantly among themselves irrespective of the media on which they were grown (Table 4). Medium composition was also had significant effect where MEA followed by PDA were most suitable for their growth. The results as presented in Table 4 showed that on PDA, the growth rate between 7th to 9th day of inoculation ranged between 9.70-15.43 mm/day and the highest was observed in PSC4m followed by PSC2m. Malt Extract Agar (MEA) was significantly better than PDA, where the growth rate from 7th to 9th day of inoculation varied between 12.78-15.24 mm/day. Highest growth rate on MEA was found in PSC1m. V8 Juice agar medium was significantly at par with PDA, where the growth rate was ranged between 10.43 to 13.59 mm/day, being found maximum in PSC1m. In OMA, the mean growth rate was 8.42 mm/day, whereas, in CDA minimum response was observed with the growth rate from 7th to 9th day of inoculation and ranged between 5.54-7.33 mm/day. In most of media tested, PSC5m was the monokaryotic strain having lowest growth rate. From the above screening, one fastest growing strain (PSC1m) and one slowest growing strain (PSC5m) were selected for hybridization.

3.2 Selection of Medium for Hybridization

It was clear from the above results that PDA and MEA were best suited media for monokaryotic strains of *P. flabellatus* whereas, in case of *P. sajor caju* it was MEA. The average growth rate of both species of *Pleurotus* on different media was compared and MEA was found to be the best medium for interspecific hybridization between selected monokaryotic strains of *P. flabellatus* and *P. sajor caju,* showing growth rate of 13.66 mm/day between 7th to 9th day of inoculation followed by PDA with 12.83mm/day (Fig. 1). Therefore, MEA was opted for performing the hybridization process.

Table 3. Effect of different media on growth performance of homokaryons of *P. flabellatus*

Isolates	Growth rate (mm/day) between 7th-9th day of inoculation on different media					
	PDA	CDA	V^8JA	MEA	OMA	Mean
PF1m	14.59	5.19	10.69	14.63	9.35	10.89^{CDE}
PF2m	14.52	4.35	10.28	14.31	10.37	10.77^{DE}
PF3m	14.89	4.81	11.61	14.44	10.11	11.17^{CD}
PF4m	13.48	6.00	10.85	15.52	11.59	11.49^{BCD}
PF5m	15.69	4.70	10.15	15.07	10.54	11.23^{CD}
PF6m	14.91	7.11	10.37	13.78	11.02	11.44^{BCD}
PF7m	9.52	4.09	6.69	9.50	8.00	7.56^{F}
PF8m	9.93	4.06	6.41	9.39	7.81	7.52^{F}
PF9m	17.15	9.48	11.37	14.69	12.56	13.05^{A}
PF10m	16.48	7.35	10.93	14.22	13.00	12.40^{AB}
PF11m	14.93	4.74	12.50	15.72	11.74	11.93^{BC}
PF12m	14.02	4.17	11.83	15.00	11.13	11.23^{CD}
PF13m	8.98	3.06	5.13	10.63	8.46	7.25^{F}
PF14m	13.37	3.81	8.46	13.61	9.98	9.85^{E}
PF15m	10.26	3.33	8.80	9.07	6.81	7.66^{F}
Mean	13.51^{A}	5.08^{C}	9.74^{B}	13.31^{A}	10.17^{B}	
	Isolate		Media		Isolate × Media	
LSD o.o5	0.94		0.61		2.36	

Table 4. Effect of different media on growth performance of monokaryons of *P. sajor caju*

Isolates	Growth rate (mm/day) between 7th-9th day of inoculation on different media					
	PDA	CDA	V8 Juice Agar	MEA	OMA	Mean
PSC1m	11.43	7.33	13.59	15.24	9.70	11.45^{A}
PSC2m	12.24	6.76	12.02	14.61	9.35	10.99^{B}
PSC3m	9.70	6.43	11.87	14.43	8.19	10.12^{C}
PSC4m	15.43	6.11	11.11	13.06	7.63	10.66^{B}
PSC5m	11.98	5.54	10.43	12.78	7.24	9.59^{D}
Mean	12.16^{B}	6.43^{D}	11.80^{B}	14.02^{A}	8.42^{C}	
	Isolate		Media		Isolate × Media	
LSD 0.05	0.39		0.39		0.86	

Hernandez and Salmones [6] had obtained 16 strains of *P. ostreatus* by interbreeding in which selection was done on the radial growth rate of monokaryotic mycelia. He found that the biological efficiency of mushroom depends on the development of mycelia in the first cultural stage. Mycelium growth of Pleurotus depends on several factors such as growing media, different media concentration, pH, temperature, nutrient element and some environmental factors [7]. Stanley and Nyenke [8] studied the cultural variability of *P. pulmonarius* in selected culture media and observed the colony diameter was highest in MEA which was 7.5 cm and in PDA it was only 4.4 cm after the 9th day of incubation. Malt extract agar media as one of the suitable cultural media for the growth of edible fungus was reported by Kalm and Kalyoncu [9]. This result gave evidence of Quimio [10] who obtained accelerated mycelia growth of *Auriculari* spp. due to incorporation of malt and rice bran extracts in agar.

3.3 *In vitro* Evaluation of Interspecific Hybrids of *P. flabellatus and P. sajor caju.*

Twenty inter specific hybrids were tested for their growth on MEA under *in vitro* condition. Significant variations in growth rate on 6th days of inoculation among the hybrids were observed (Fig. 2). Growth rate was observed higher in hybrid PF×PS7 (16.40 mm/day) which was at par with the growth of other three hybrids *viz*. PF×PS17, PF×PS19 and PF×PS13 (16.29 mm/day, 16.18 mm/day and 16.01 mm/day, respectively). Lowest growth rate was found in PF×PS1 (11.61 mm/day).

Plate 3. Isolation of spore through serial dilution

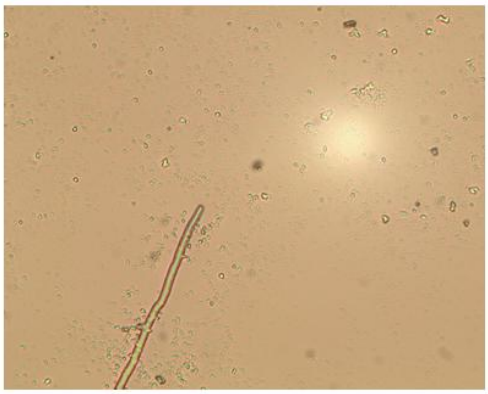


Plate 4. Hyphal tip without clamp connection for isolation

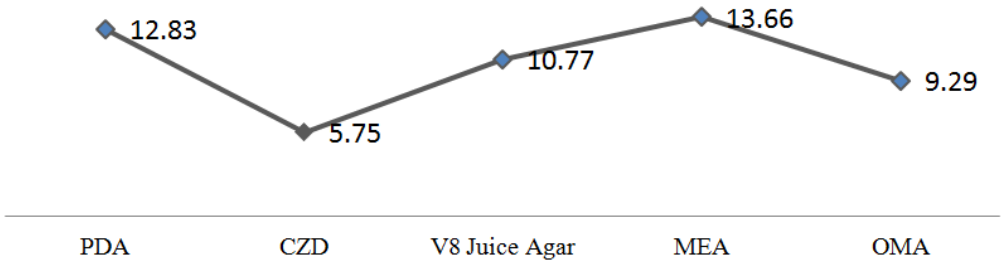


Fig. 1. Screening of medium for inter and intraspecific hybridization of *P. flabellatus* and *P. sajor caju*

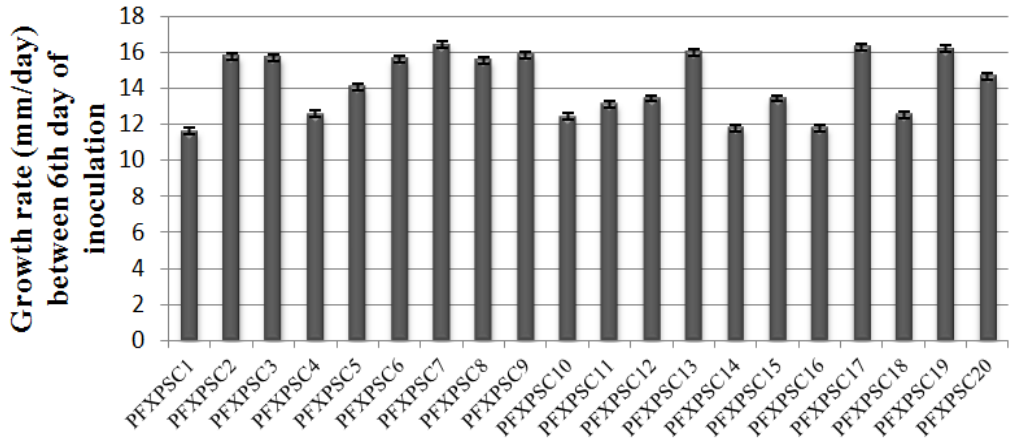


Fig. 2. *In vitro* growth of interspecific hybrids of *P. flabellatus* and *P. sajor caju*

3.4 *In vitro* Evaluation of Intraspecific Hybrids of *P. flabellatus*

Thirty four intra specific hybrids were then evaluated on MEA medium for 6 days of inoculation for identifying the strains with higher growth potential. The results revealed that the intraspecific hybrids *viz.* PF×PF27, PF×PF10, PF×PF33, PF×PF24, PF×PF5, PF×PF25, PF×PF4, PF×PF12, PF×PF1, PF×PF26 and PF×PF13 exhibited mycelial growth rate above16mm/day and were screened preliminarily as promising hybrids having good growth rate.

It is evident from the results of *in vitro* that mycelia growth rate of both inter and intraspecific hybrids of *Pleurotus* sp were more as compare to the momokaryotic isolates. The present result was in agreement with the result of Bahukhandi and Sharma [5] who observed that the single spore isolate were having slower growth and in some case the growth was limited within the inoculum. Peng [11] had selected the 92 hybrids dikaryons by taking mycelial growth rate as important parameter to examine its fruit bodies from which four highly productive strains were obtained.

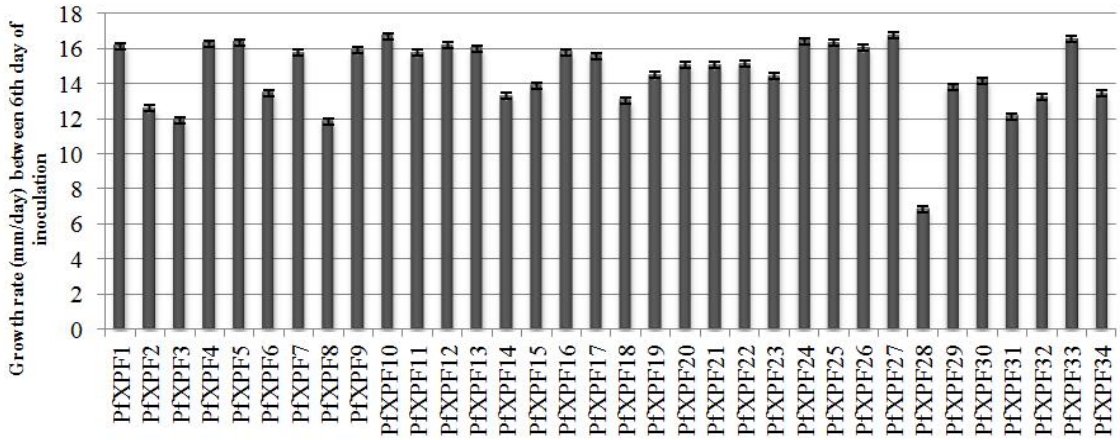


Fig. 3. Screening of intraspecific hybrids of *P. flabellatus* based on *in vitro* growth potential

Plate 5. Monosporus cultures of *P. flabellatus*

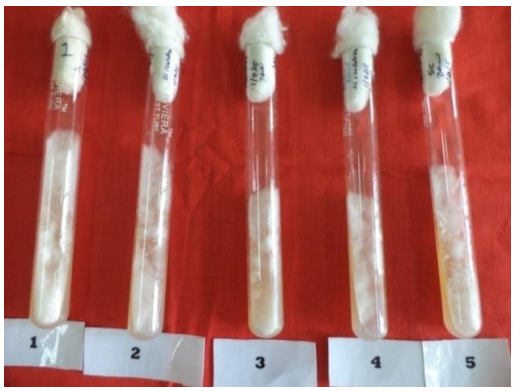


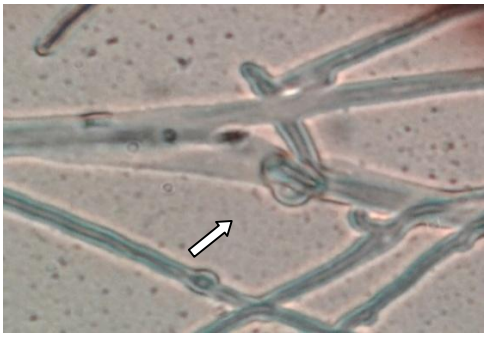
Plate 6. Monosporus cultures of *P. sajor caju*

Plate 7. Variation in growth of homokaryons of *P. flabellatus* on different media

Plate 8. Variation in growth of homokaryons of *P. sajor caju* on different media

Plate 9. Somatic hybidization in Pleurotus

Plate 10. Isolation of hybrids from meeting point



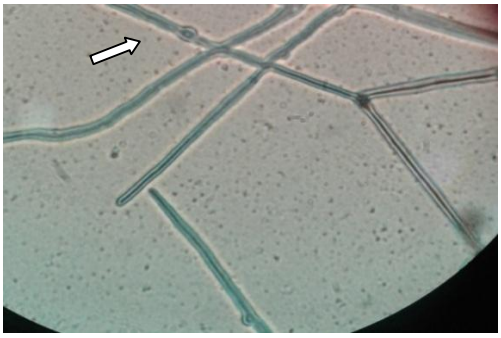


Plate 11. Microscopy for presence of clamp connection to ascertain hybridization

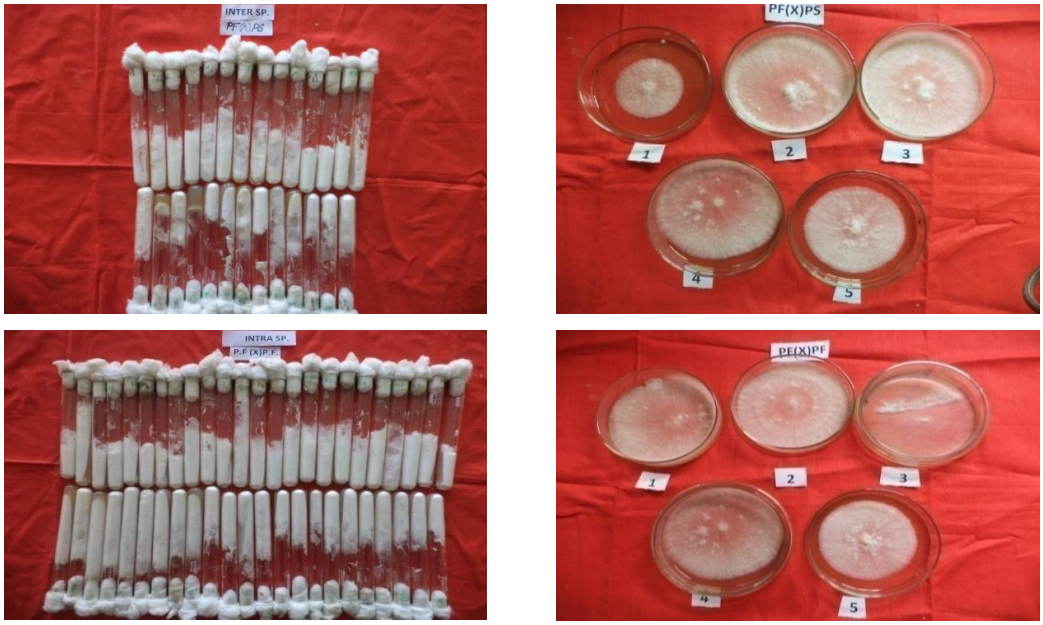


Plate 12. Growth test of interspecific hybrids of *P. flabellatus* and *P. sajor caju*

Plate 13. Growth test of IntraSpecific hybrids of *P. flabellatus*

4. CONCLUSION

Variations in growth rate were observed in monokaryotic isolates, MEA medium was found to be a suitable medium for dikaryotization of *P. flabellatus* and *P. sajor-caju*. Dikaryotic mycelium showed the highest growth rate of above 16 mm/day as compare to monokaryotic isolates of *P. flabellatus and P. sajor-caju*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23933