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Development, evaluation and characterization of browning-resistant hybrids of white button mushroom (*Agaricus bisporus*)

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Abstract

Postharvest browning in white button mushroom is a major limiting factor determining the quality and marketability of mushrooms. In the present study, 361 hybrids were developed using non-fertile isolates from eleven button mushroom strains. A total of forty one browning tolerant hybrids were selected and evaluated for the yield. Five of these were evaluated on large scale in commercial units and two have been finally selected on the basis of higher yield than the strains used by the commercial units. The selected five hybrids showed very low level of enzymes responsible for browning i.e., laccase, polyphenol oxidases and tyrosinase.The hybrids were characterized using SSR, IRAP and ReMAP markers and distinguishing bands for hybrids could be identified. The developed hybrids will address post harvest browning problems of button mushroom industry.

Keywords: *Agaricus bisporus*, hybrids, browning resistance, yield evaluation, molecular characterization

Introduction

The white button mushroom, *Agaricus bisporus*, is highly popular throughout the world contributing almost 15% of the total world production of mushrooms (Royse 2016). The marketability of button mushrooms very much depends upon its natural white colour and browning resulting from bruising during handling and transport decreases its commercial value. Mushroom browning caused by mechanical damage is a consequence of the enzyme-catalyzed oxidation of phenols into quinones and then into dark melanins (Jolivet et al. 1998). The study of Esquerre et al. (2009) confirms that release of water due to physical damage causes activation of tyrosinase enzyme, catalyzing oxidation of phenolics to quinones. To maintain the mushroom quality after harvesting, there is need to select/breed superior strains that are less sensitive to mechanical damages.

The breeding of white button mushroom (*A. bisporus*) is a complicated proposition because of its unusual secondary homothallic sexual behavior (Raper et al. 1972) and needs monokaryotic spores for hybrid breeding. The only reliable method to identify self-sterile monokaryotic isolates is the fruiting trial. The studies of Gao et al. (2015) and Eastwood et al. (2001) showed that the browning in mushroom is a genetically controlled character and targeted cross breeding can be one important strategy to overcome this problem. The first hybrid in button mushroom was developed in 1976 at Mushroom Experimental Station, Horst, The Netherlands, the U1 and its segregant U3. Since then not many new hybrids with different genetic background have been developed and all currently grown cultivars are related to a limited number of traditional genotypes. Breeding programme in India started in 1984 (Bhandal and Mehta 1989) and the first hybrid NCH 102 was released in 1997.

During the present study, 39 self-sterile spores from 11 genetically diverse parents were identified through fruiting trials. These self-sterile spores were mated in different combinations to generate hybrids and confirmed through fruiting trials with a view that some of the combination may have non-browning characteristics. The browning sensitivity of mushrooms was screened and analyzed along with yield. The aim of this study was to develop high yielding browning-resistant hybrids in *A. bisporus* for commercial sustainability of the mushroom cultivation.

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Materials and methods

Germplasm used

A total of 1000 single spores were isolated from eleven strains, namely, S-11, U-3, A-4, A-6, A-16, A-2, A-15, A-94, S-465, S-130, and a wild strain WI-1. All the strains used in the study were obtained from culture collection of ICAR-Directorate of Mushroom Research, Solan, and Indian Agriculture Research Institute, New Delhi.

Single spore isolation and their cultivation

All the strains were cultivated on pasteurized compost using standard cultivation practices to collect the spore prints for single spore isolation. Single spores were isolated using serial dilution technique on the standardized media containing dextrose – 5.0 g; MgSO4 –0.25 g; KH2PO4 – 0.95 g; succinic acid – 1.25 g; Agar –15.0 g; water 500 mL and pH –5.0. Placing *Agaricus* mycelium on the lid triggered the spore germination. The spawn of a total of 1000 isolated single spores was prepared on wheat grains following standard practice and was cultivated on pasteurized compost in 10 kg bags. The yield of the each bag was taken up to four weeks and then average for each SSI was calculated.

Intermating of homokaryons

Self-sterile single spore isolates were used in the hybridization experiments. Homokaryotic mycelia were grown side by side on standardized modified CYM medium containing glucose 20g; peptone –2 g; yeast extract – 1g; MgSO4.7H2O – 0.5g; KH2PO4 – 1.0g; Agar – 20g and distilled water: 1000mL. The pH of the medium was kept 6.5. The mycelium from the contact zone was picked and tested for fruiting to confirm the hybrid status as no reliable marker is available for identification/confirmation of hybrid status in *A. bisporus.*

Cultivation trial of the hybrids and test of browning resistance

Hybrids status was confirmed using fruiting trials on 100 kg compost. The confirmed hybrids were tested for browning reaction by applying double streak mechanical injury method (Weijin et al. 2012). The selected five browning-resistant hybrids were evaluated for three consecutive years at ICAR-Directorate of Mushroom Research, Solan and at five different centres of All India Coordinated Research Project on Mushrooms (AICRP-M) on 320 kg compost/hybrid. Further, two selected hybrids were evaluated at three different large button mushroom farms of 5000 TPA capacities i.e. Tirupati Balaji Agro-Products, Baramati, Pune (Maharashtra), Flex Foods, Dehradun (Uttarakhand) and Weikfield's Mushroom farm, Pune (Maharashtra). Firstly, all the five selected hybrids were evaluated on 1000 kg compost each. In second phase, the two selected hybrids were evaluated on 42 tonnes of compost and finally on 125 tonnes compost.

Enzyme assay

The fruit bodies of all the five non-browning hybrids were assayed for enzymes responsible for browning i.e. laccase, tyrosinase and poly phenol oxidase. Laccase (E.C. 1.10.3.2) was assayed by adding enzyme source to a reaction mixture (300µM NaH_2PO_4/Na_2HPO_4 buffer pH-6 and 30 µM Guiaicol) and measuring δA470 (E470- 0.487) (Turner et al. 1975). Polyphenol oxidase (E.C. 1.10.3.1) was measured following Turner (1974) as δA495 with catechol as substrate. For tyrosinaseassay, δA470 was read in reaction mixture containing 2.5 mM of L-Dopa in 0.1 M phosphate buffer (pH-7.4) and 100 mL enzyme source (Miranda et al. 1988). The molar extinction coefficient of Dopa is 3313 M^{-1} eM^{-1} (E_{470} = 33.13).

Statistical analysis was done for randomized block design using one-way ANOVA to calculate standard error and the least signiûcant difference (LSD). The data presented in the table contains standard error and the least significant difference (LSD) (5%).

DNA isolation, PCR amplification and sequencing of ITS regions

Genomic DNA was extracted from liquid nitrogen dried mycelium grown on malt extract broth medium (malt extract 10 gL^{-1} and dextrose 5 gL^{-1}) as per Punja and Sun (2001). The DNA concentration was estimated using a spectrophotometer taking optical density at 260 and 280 nm. The final working concentration of DNA was standardized to 50 ng μl^{-1}. The PCR primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White et al. (1990) were used to amplify the ITS region of ribosomal DNA. Amplifications were done following Singh et al. (2004). The PCR products were visualized on 1.6% agarose gel in 1x TAE buffer. PCR products were directly sequenced using automated sequencer of Applied Biosystems (3730 analyser) using ITS-1 and ITS-4 primers.

Genotyping of SSIs, hybrids and their parents

The SSIs and hybrids were genotyped along with their parents for diversity analysis and developing markers for confirmation of hybrids using RAPD, microsatellite (ISSRs and SSRs) and retro-element based markers (IRAPs and ReMAPs). A total of 50 RAPD, 7 ISSR, 33 SSRs, 29 outward facing IRAP and 9ReMAP primers were used.

RAPD amplifications were performed following Singh et al. (2004) while ISSR amplifications were performed following Nazrul and Yin Bing (2011). For SSR, the amplifications were performed following Foulongne-Oriol et al. (2009). IRAPs and ReMAP amplifications were performed following Teo et al. (2005).

Bioinformatic analysis

The complete sequence data (ITS-1, 5.8s and ITS-4) were compared with NCBI database using BLAST search. The sequences were subjected to further analysis using Mega 6.0 software (Tamura et al. 2013) for maximum likelihood analysis with 1000 bootstrap comparisons. The sequence data were further subjected to Bayesian analysis to calculate the posterior predictive distribution to do predictive inference by Mr Bayes software using Markov Chain Monte Carlo (MCMC) Methods (Ronquist and Huelsenbeck 2003).

The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the RAPD, ISSR, SSR, IRAP and ReMAP profiles were analysed using NTSYSpc software 2.02 (Nei and Li 1979). To validate the analysis, bootstrap analysis was performed using Winboot software keeping 1000 bootstrap comparisons.

Results

Thirty-four button mushroom strains including a wild strain were subjected to diversity analysis using RAPD and ISSR markers. The RAPD markers revealed very low diversity while the ISSR markers showed higher diversity (Fig. 1). On the basis of diversity analysis eleven *A. bisporus* strains namely, A-2, A-4, A-6, U-3, A-94, S-465, A-15, A-16, S-130, S-11 and a wild strain WI-11 were chosen for this study. Out of 1000 SSIs from the selected strains, 39 SSIs were found non-fruiting and remaining were fertile. The SSIs that started fruiting after first flush were taken as false positives and were considered as non-fertile isolates. ITS 5.8S rDNA sequences were used to confirm the identity and diversity amongst SSIs (Figs. 2 and 3). The variability amongst non-fertile and fertile SSIs was also analyzed using RAPD and ISSR markers. RAPD exhibited a very low polymorphism (8.0%) (Figs. 4 and 5). The ISSR markers revealed higher genetic diversity (Figs. 6 and 7) of 66% in non-fertile isolates than amongst fertile isolates (30%).

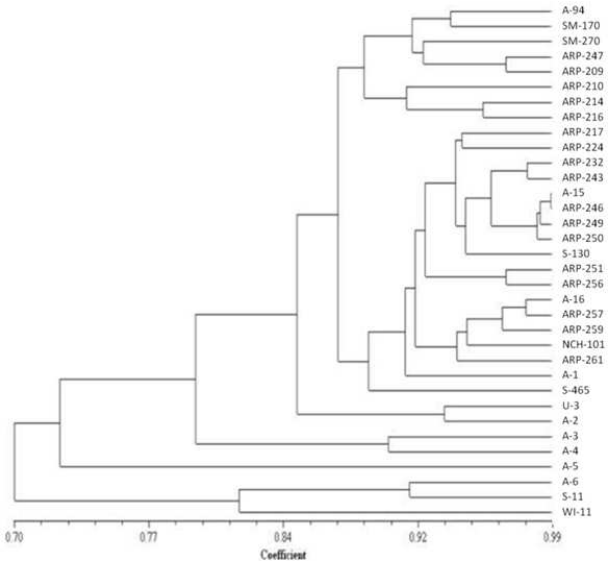

Fig. 1. Diversity amongst different strains of *Agaricus bisporus* based on ISSR polymorphism

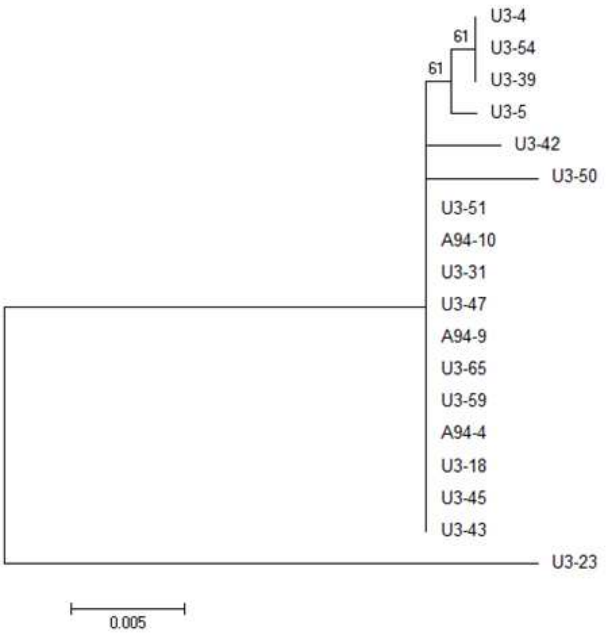

Fig. 2. Diversity amongst 18 SSIs of *A. bisporus* based on ITS 5.8S rDNA sequences using the Maximum Likelihood method in MEGA6

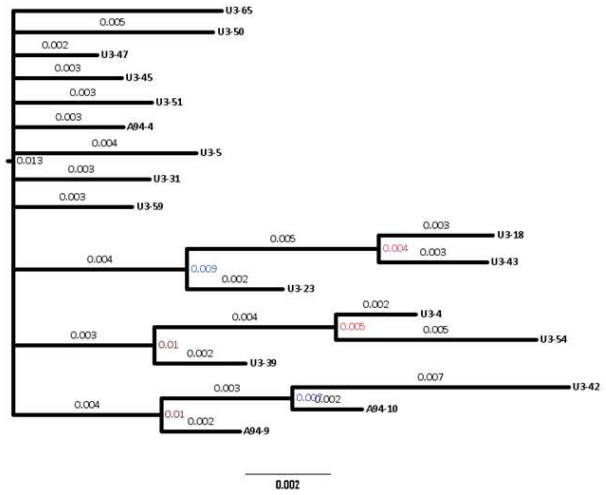

Fig. 3. The Bayesian analysis of the phylogenetic tree of *A. bisporus* SSIs generated by Mega 6

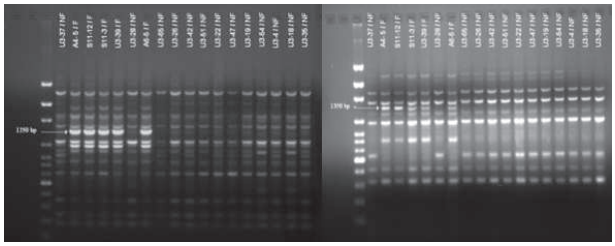

Fig. 4. RAPD gel photographs showing very low diversity amongst the single spore isolates of button mushroom (Specify the RAPD primer name against each photo)Fig. 5. Diversity among18 SSIs of *A. bisporus* based on RAPD polymorphism

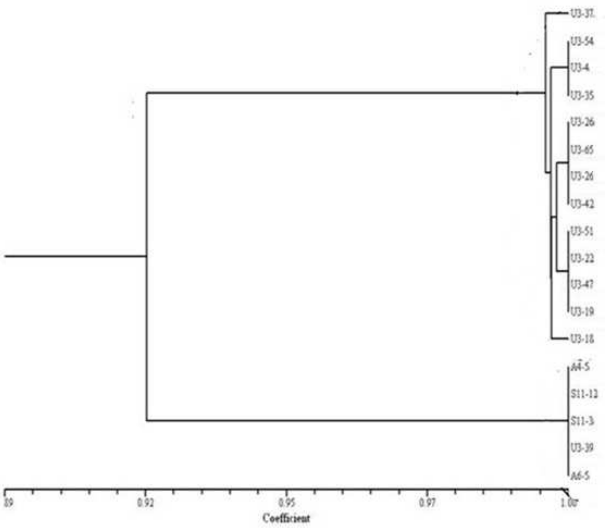

Fig. 5. Diversity among 18 SSIs of *A. bisporus* based on RAPD polymorphism

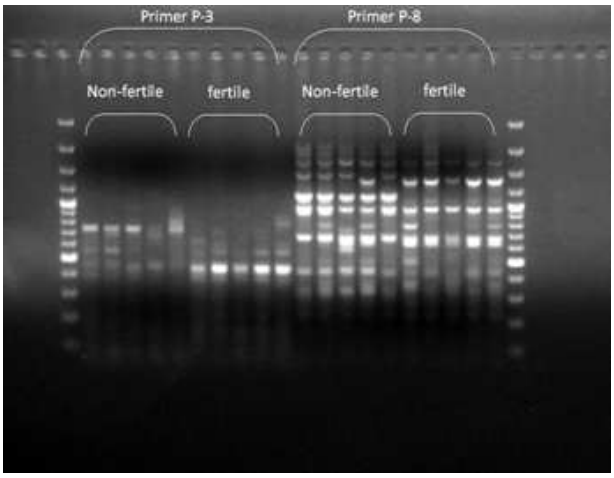

Fig. 6. ISSR profile of 5 fertile and 5 non-fertile isolates using P-3 and P-8 primers

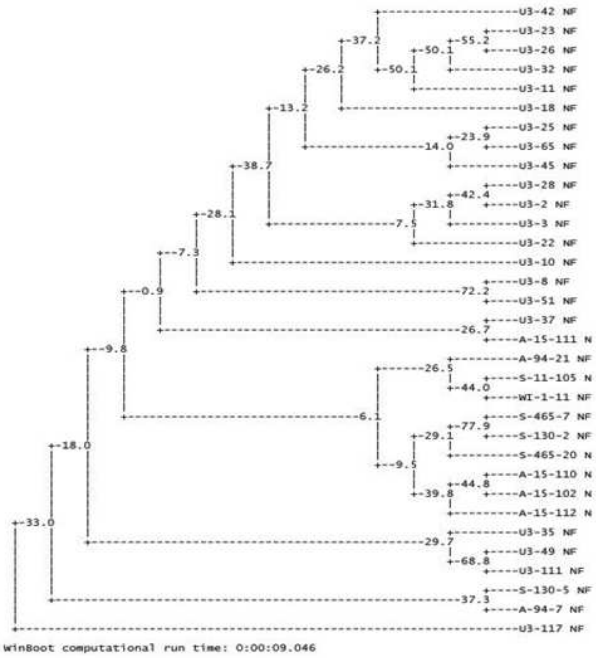

Fig. 7. Diversity amongst non-fertile single spore isolates using ISSR markers

A total of 35 non-fertile isolates were taken for inter- and intra-strainal hybridization (Fig. 8). Out of 441 putative hybrids, 161 showed fertility in fruiting trials confirming the hybrid status. A total of 41 hybrids were selected on the basis of browning resistance, out of which 5 hybrids were further selected on the basis of higher browning resistance and yield potential. The selected five hybrids almost showed negligible browning even after 2 hours of mechanical injury (Figs. 9 and 10).

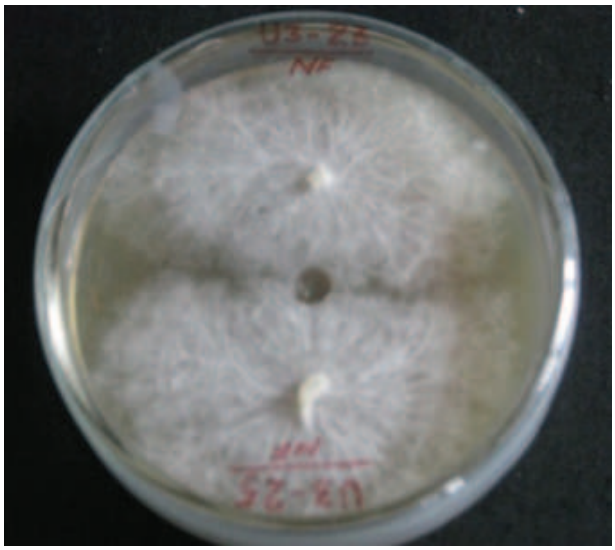

Fig. 8. Hybridizing non-fertile isolates

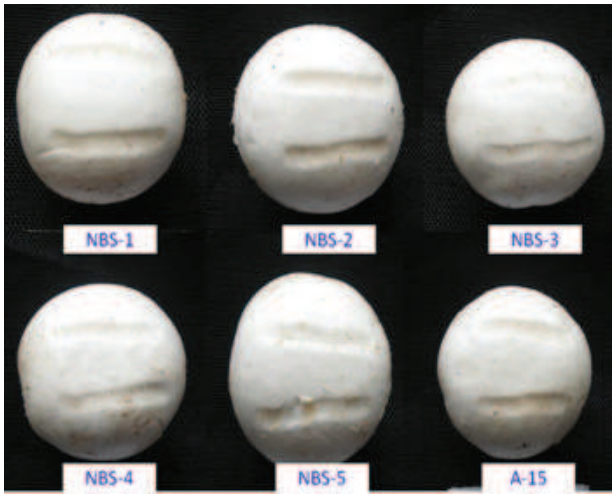

Fig. 9. Browning reaction after two hours using double streak mechanical injury

Fig. 10. No browning even after 2 h of cutting: upper=NBS-1; lower=NBS-5

The selected hybrids named as NBS-1 to NBS-5 were evaluated at ICAR-DMR, Solan (Table 1), AICRP-M (Table 2) and at three commercial farms (Table 3). Both the hybrids, NBS-1 and NBS-5, gave

Table 1. Performance of selected high yielding non-browning hybrids (NBS-1 and NBS-5) at DMR, Solan in preliminary trials

Strains	Average yield (kg/100 kg compost)		
	2012-13 (on 150 kg compost)	2013-14 (on 500 kg compost)	2014-15 (on 500 kg compost)
NBS-1	18.90	18.50	17.90
NBS-5	15.75	17.80	16.27
Control (U-3)	15.40	14.00	14.65
LSD (0.05)	1.12	0.81	1.22

Table 2. Performance of selected non-browning hybrids of white button mushroom in four weeks cropping at different locations during 2014-16

Strains	Average yield (kg/100 kg compost)*					
	Pant-nagar	Pune	Nauni	Ludhiana	Solan	Aver-age
NBS-1	20.27	17.17	20.50	16.35	18.19	18.27
NBS-5	19.79	18.17	16.27	—	15.50	17.65
Control (U-3)	19.79	19.05	16.65	10.54	15.50	15.28
LSD (0.05)	1.22	0.81	0.86	0.81	1.12	

*each strain tested on 320 kg compost

significantly higher yields than the control strains U-3 and A-15. The hybrids yielded 10-15 per cent higher than the imported strains at these mushroom farms (Figs. 11 and 12). The fruit bodies of all the five non-browning hybrids were assessed for phenol content and enzymatic activity related to browining, namely, laccase, tyrosinase and polyphenol oxidase. Only two hybrids (NBS-1 and NBS-4) showed significantly lower phenol content than the control. However, all hybrids

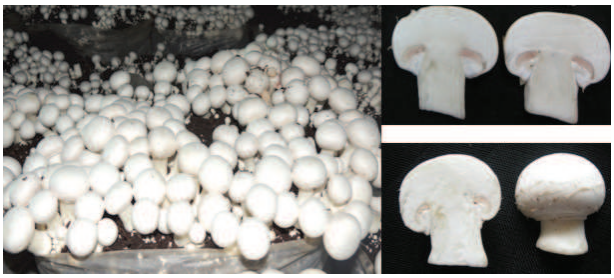

Fig. 11. Crop of NBS-1 (Left), cross section of fruit body (top right) and quality of fruit bodies after 48 h of storage at room temp (bottom right)

Table 3. Yield of NBS-1 and NBS-5 on three commercial farms during 2013-14 and 2014-15

Commercial farm	Hybrids	2013-14	2014-15	2015-16	Average
Tirupati Balaji Agro-products, Baramati (Maharashtra)	NBS-1	25.19	19.13	18.92	21.08
	NBS-5	24.16	21.80	25.52	23.82
	Control (Ital Spawn)	22.95	18.16	22.33	21.14
Weikfield Mushroom, Pune (Maharashtra)	NBS-1	21.26	22.00	—	21.63
	NBS-5	23.06	16.70	—	18.70
	Control (A-15)	18.73	17.15	—	17.94
Flex Food, Dehradun (Uttarakhand)	NBS-1	—	17.58	—	17.58
	NBS-5	—	18.30	—	18.30
	Control (A-15)	—	16.28	—	16.28

*The data of Tirupati Balaji Agro-products during 2015-16 is average of 10 lots of compost of 125 tons each lot

Table 4. Phenol content and enzyme activity of browning-resistant hybrids of button mushroom

Hybrids	Phenol (µg/g fresh mushroom)	Laccase*	Tyrosinase*	Polyphenol oxidase**
NBS-1	166.36	0.053	9.66	0.97
NBS-2	230.10	0.046	15.99	1.23
NBS-3	220.69	0.025	16.76	1.40
NBS-4	171.44	0.033	13.26	1.44
NBS-5	168.36	0.035	9.51	1.43
A-15	220.16	0.137	18.28	2.17
LSD (5%)	19.00	0.027	3.46	0.24
Standard Error	8.53	0.012	1.55	0.11
Coefficient of variance (%)	5.32	27.470	13.69	9.24

* = δA_{470} by 0.001/min/µg protein; ** = δA_{495} by 0.001/min/µg protein

Fig. 12. Crop of NBS-5 (left), cross section of fruit body (top right) and quality of fruit bodies after 48 h of storage at room temp (bottom right)

showed nearly 50% reduction in laccase and polyphenol oxidase activity than the control (Table 4).

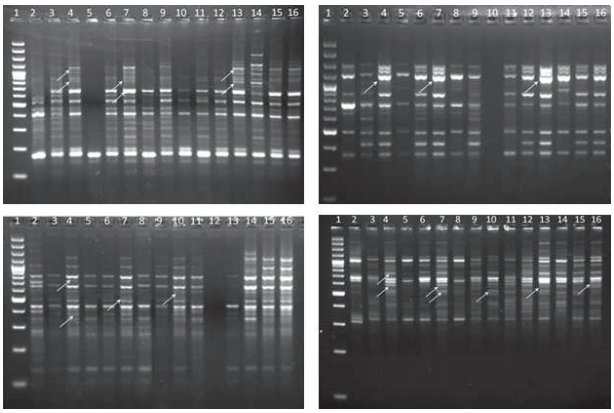

Fig. 13. Determining hybridity using molecular markers (lane1- DNA ladder, lanes 2 and 3-parents, lane 4-hybrid, lanes 5 and 6-parents, lane 7-hybrid; lanes 8 and 9-parents; lane 10-hybrid, lanes 11 and 12-parents, lane 13-hybrid, lanes 14 and 15-parents, lane 16-hybrid)

Further, the hybrids were molecularly characterized along with their parents for developing markers for confirmation of hybrid and strain protection using ISSR, SSR, IRAP and REMAP markers. A total 585 markers were scored for presence/absence of bands. Genetic divergence of hybrids ranged between 8% and 23% from their parental strains with strong bootstrap values (Fig. 14). SSR and retro element based markers very well differentiated and showed clear resolution in identification of hybrids (Fig. 13). It was also observed that all the hybrids were genetically closer to one of the parents, which came from the U-3 genotype.

Discussion

The enzymatic discolouration of mushrooms and other

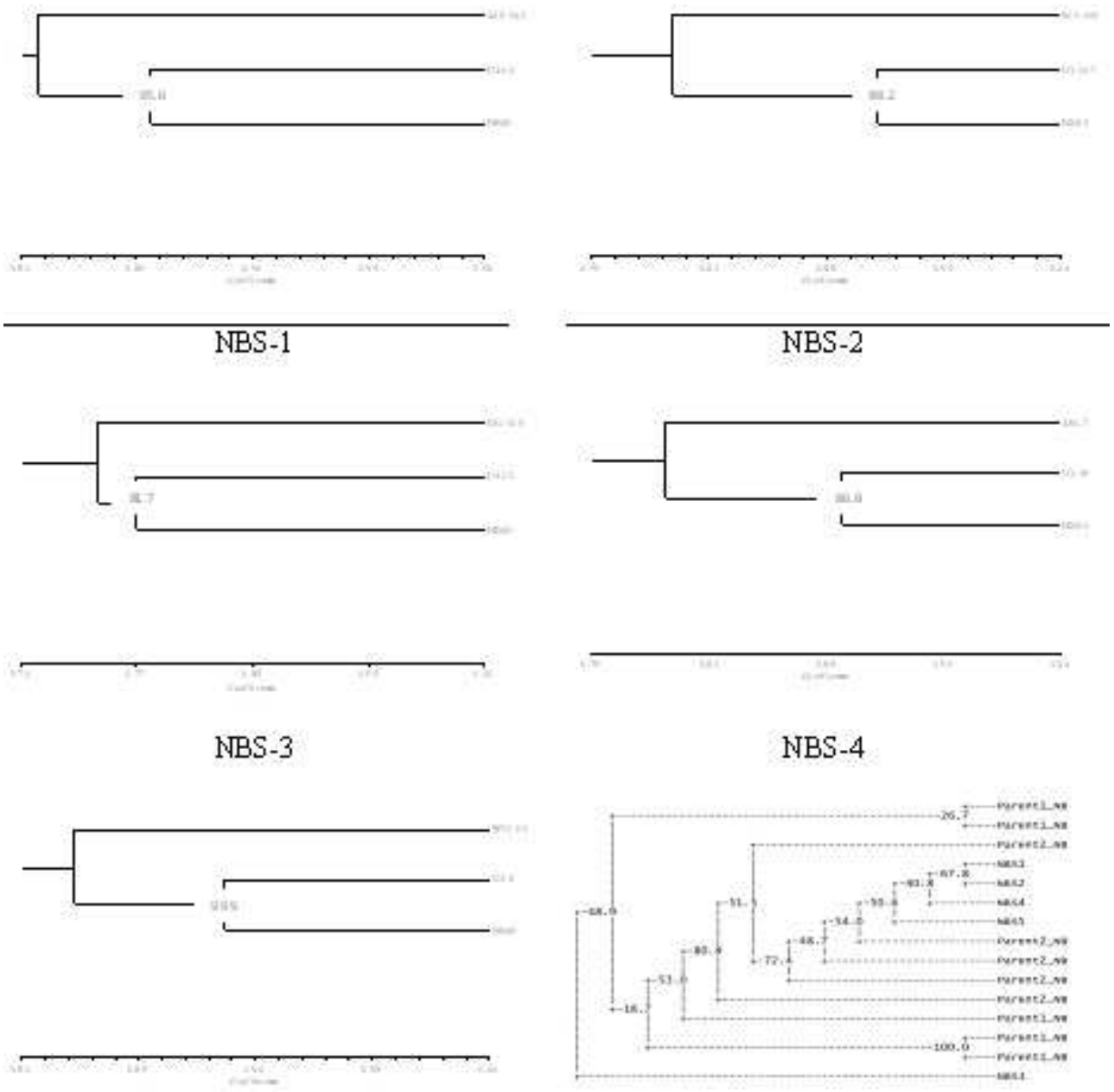

Fig. 14. Diversity analysis in gel profiles of SSR, ISSR, IRAP and ReMAP markers in the hybrid NBS-1 to NBS-5 and their parental strains using NTSys PC Ver 2.02 and bootstrap analysis was done using Winboot for binary data analysis

crops is largely mediated by copper oxygenases called polyphenol oxidases (PPOs: laccases and tyrosinases) and peroxidases. In button mushroom browning, tyrosinase plays the most important role (Turner 1974). Weijn et al. (2012) observed varying bruising sensitivity in different strains of *A. bisporus*. Studies of Gao et al. (2011) indicated that brown wild strains showed less bruising sensitivity than white

commercial lines. Genetic analysis of the trait by Gao et al. (2015) indicated that bruising sensitivity is a polygenic highly heritable trait (H2: 0.88-0.96). Eastwood et al. (2001) identified 20 genes with increased expression in 2-day postharvest mushrooms.

During the present study, we could develop browning-resistant hybrids NBS-1 and NBS-5 by mating two genetically diverse strains, of which one of the parents was invariably derived from strain U-3. This clearly indicates that strain U-3 (Fritsche 1983) contains the character of browning resistance, which might have been partly masked due to polygenic condition. Meotic recombination of alleles may have resulted in unmasking of those genes in newly developed button mushroom hybrids.

The multinuclear mycelia, absence of clamp connections and the absence of asexual spores in *A. bisporus*, make it difficult to generate mutants and hybrids (Sarazin 1952; Evans 1959). Therefore, developing diagnostic markers (classical/ morphological/DNA based) that can help to categorize single spore isolates into monokaryotic (non-fertile) and dikaryotic (fertile) type is critical for developing hybrids. This will avoid the laborious and time consuming fruiting trials and will simplify the breeding process.

For any successful breeding programme, genetic diversity and wide gene pool is of paramount importance. In the present study, low genetic diversity was revealed by RAPD markers while ISSR and SSR markers showed higher genetic diversity. In mushrooms, RAPDs have been used for diversity assessment, screening of homokaryotic lines and confirmation of hybridization (Khush et al. 1992; Sunagawa et al. 1995). Chillali et al. (1998) used analysis of ITS and IGS regions for evaluation of genetic diversity. Retro elements have been used as genetic markers to investigate diversity and phylogeny (Bennetzen 2000). In particular, the IRAP technique developed by Schulman et al. (2004) has been useful to provide multi-locus anonymous markers.

Traditional hybridization techniques was used for hybrid breeding. Kneebone et al. (1972) were the first to attempt hybridization in mushroom by growing strains together on grains while Stoller (1974) attempted chemically (nicotinic acid) induced mycelium fusion in submerged cultures. Sinden (1981) and Fritsche (1983) attempted to obtain hybrids by strain mixing. Till date the only method to confirm the hybrid status is the fruiting trials, which are laborious and time consuming and restricts the breeding efforts. In this study, molecular markers (ISSRs, SSRs, IRAPs and ReMAPs) were used to characterize the hybrids along with their parental genotypes to identify the markers for establishing the hybrid status. Various studies have reported use of molecular markers such as RFLP, ISSR and AFLP for confirmation of hybridity and for differentiating hybrid strains (Horgen and Anderson 1989, Ghorbani Faal et al. 2009; Guan et al. 2008; Malekzadeh 2011). Results of all earlier studies showed a high similarity coefficient between the strains and strain U1. Le et al. (2008) demonstrated the effectiveness of REMAP markers in mushroom fingerprinting.

In the present study, browning-resistant hybrids were developed through cross breeding technique between the homokaryotic lines obtained from the diverse parents of button mushroom. The selected hybrids were tested at a commercial level for yield and browning. The two selected hybrids NBS-1 and NBS-5 were released after three years evaluation at different scales. Also, the hybrids were molecularly characterized for identification of markers and confirmation of hybridization. This study will help the button mushroom industry that is suffering from the post harvest browning problems in button mushroom due to handling and picking. This study is also important from the point of view that some markers are identified to confirm the hybrid status and will hasten the breeding procedure in button mushroom.

Authors' contribution

Conceptualization of research (MS); Designing of the experiments (SK); Contribution of experimental materials (SK); Execution of field/lab experiments and data collection (SK, MG, AB); Analysis of data and interpretation (SK, AB); Preparation of manuscript (SK, VPS).

Declaration

The authors declare no conflict of interest.

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