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Enzyme profile of Shiitake mushroom strains grown on wheat straw

Sudheer Kumar A*, V.P. Sharma, Satish Kumar, Anupam Barh, Sunny Banayal and Shwet Kamal

ICAR-Directorate of Mushroom Research, Solan 173213, Himachal Pradesh

ABSTRACT

The mycelial growth abilities and extra cellular enzyme activities of nine different strains of shiitake cultivated on wheat straw were measured to establish predictable information on strain-substrate compatibility. The experimental results showed that the strains varied significantly in their mycelial growth rate, enzyme activities and in turn the biological efficiency. Fast mycelial colonization and higher amount of cellulase activity was observed in strain DMRO-327 with the highest mean fresh mushroom yield of 202.32 g per kg of wetted substrate. Correlation study was found significant between the initial speed of linear growth rate and biological efficiency of various strains. The rise in activity of carboxy methyl cellulase, FPase and xylanase during the primordial formation illustrated the role of these enzymes to promote fruiting of shiitake in straw based substrate. While decrease in the lignolytic enzymes with the time showed that these enzymes are responsible for substrate degradation. The conspicuous variations noticed in the morphometric observations also indicated the strain preference towards the given substrate.

Key words: *Lentinula edodes*, selection of strains, alternate substrates, extra cellular enzyme activity.

INTRODUCTION

Shiitake mushroom, {*Lentinula edodes* (Berk) Pegler} has been a highly rated delicacy in South East Asian countries such as China, Japan, Taiwan, Korea etc. People from orient have enjoyed shiitake mushroom for ages as folk medicine. The interest in consumption of shiitake is constantly rising because of its exotic flavour, taste and enticing aroma. Besides these culinary properties, the nutritional and medicinal attributes made the shiitake as the second most widely grown edible mushroom in the world (Qi *et al.,* 15). The steadily increasing demand for shiitake at global level opened the market outside Asia and created a scope for extensive cultivation beyond oriental region. Traditionally, shiitake mushrooms are cultivated on natural logs collected from the hardwood trees, but the commercial cultivation of shiitake gained momentum with an advent of synthetic log cultivation, prepared by enriched sawdust (Kalberer, 7).

Even though, the short duration cultivation technology of shiitake under indoor conditions was standardized (Sharma *et al.,* 19), still this valued mushroom has so far not been exploited at commercial scale in India. The prime constraint behind its poor adoption is non availability of good quality sawdust from desired tree species. Since, edible mushrooms are adopted to grow on a wide range of lingo-cellulosic wastes, it is necessary to explore the suitability of alternative agro residues available in abundance for the commercial cultivation shiitake. Out of the various agricultural residues, availability of wheat straw is 113 million tons in India (Mishra *et al.,* 12) showing great potential for its exploitation in shiitake production. The possibility of growing shiitake on wheat straw was earlier studied by several researchers

(Gaitan-Hernandez *et al.*, 5; Levanon *et al.*, 9 and Delpech and Olivier, 3). However, no study has been reported higher yields on straw based substrate, which is not economically viable to take up the production at commercial scale. Due to the limited genotypes of shiitake having ability to produce fruit bodies on straw based substrates, not much progress has been made in this area. In view of these limitations, studies were conducted to identify the promising strains adapted to grow on wheat straw by studying the growth patterns and extracellular enzyme activities at different growth stages. Subsequently, cultivation trials were conducted to explore the yield potential of the strains on cellulose rich substrate such as wheat straw.

MATERIALS AND METHODS

A total number of 35 strains of shiitake were obtained from the gene bank of the ICAR-Directorate of Mushroom Research, Solan. Preliminary screening was done to test the ability of the strains to produce sporophores on wheat straw based substrate at 20 ± 2°C of fruiting temperature. Out of the 35 strains tested, nine strains which produced fruit bodies on wheat straw were further selected to test their

Corresponding author's E-mail: sudheerannepu@gmail.com

growth rates, profile of extracellular enzymes and yield potential. Mycelial growth ability of the nine strains was studied on the wheat extract agar (WEA) medium and wheat straw substrate supplemented with the wheat bran. The WEA medium was prepared by boiling 50 g of wheat straw in a liter of water, and decanted to collect the extract. The agar powder was added @ 15g L^{-1} of the above extract and then sterilized at 121°C for 90 minutes at 15 psi pressure. The mycelium disks (8 mm dia.) of different strains pre-cultivated on malt extract agar medium were inoculated into the petri dishes and incubated at 25±2ºC. Radial growth rate (cm/day) was recorded by measuring the diameter of the mycelia along with two perpendicular axes after every two days interval and presented the data on per day growth at 10 days of incubation. Linear growth rate was studied on unsupplemented straw filled in the glass tubes and supplemented straw filled in the heat resistant polypropylene (pp) bags. The mycelial discs of uniform size of different strains were inoculated in un supplemented wheat straw filled in the glass tubes to the equal length of 10 cm. Similarly, the grain spawn of different strains was inoculated into the substrate filled in pp bags @ 4% on wet weight basis. The inoculated glass tubes and bags were kept for incubation at 25±2°C. The linear growth rate (cm/day) of mycelia was measured at every seven days interval and presented the per day growth rate data at 21 days of inoculation.

A total number of five samples were drawn from the incubation room at an interval of seven (sample 1), 14 (sample 2), 21 (sample 3) days, 28 (sample 4) from the date of spawning and the last sample at the primordial formation stage (sample 5). The substrate colonized by the mycelium of different strains from each block at specified interval was collected, homogenized, lyophilized and powdered by mechanical grinding. The powder was stored at 4°C till the assay. Crude enzyme extracts were prepared by adding 0.7g of powder to 10ml of deionized water. The extract was filtered through muslin cloth to remove the solids and cold centrifuged at 12000 × g for 15 min. Then the crude enzyme extracts were used immediately for assays.

The enzyme activities of different strains was assayed in triplicate and expressed as IU g^{-1} defined as the amount of enzyme producing 1µmol of product per min per g of substrate extracted (Table 1).

The substrate required for cultivation trials was prepared by mixing wheat straw (chopped into 4-6 cm size), wheat bran and gypsum in the ratio of 80:19:1 on dry weight basis. Moisture content of the substrate was adjusted at 66%. One kg of the substrate filled in the double PP bags and sterilized in autoclave at 121°C temperature and 15 psi pressure for two hours. The spawn of different strains prepared on wheat grain was inoculated @ 4% on wet weight basis under aseptic conditions. Nine blocks were prepared for each strain and kept for incubation at 25±2°C. Once the bump formation appears on the surface of the substrate, the pp bags were peeled off and completely colonized blocks were dipped in the ice cold water (4-6°C) for 10 min as a shock treatment to induce fruiting. The cold water treated blocks were transferred to the cropping room for fructification and productivity evaluation. The temperature and relative humidity were maintained at 20±2°C and 85±5%, respectively for fruiting. The matured fruit bodies were harvested before unveiling the cap and yield was expressed in terms of biological efficiency (BE) as per cent weight of fresh mushrooms per dry weight

Table 1. Enzymes and substrates used for assay.

Enzyme	Buffer used	Substrate used	Basis for assay	Ref
Endo-glucanase (CMCase)	0.5 mL acetate buffer of pH 5.0	Carboxy methyl cellulose	Measuring reducing sugars by DNS method	Eveleigh *et al.,* 4
Exo-glucanase (FPase)	0.5 mL acetate buffer of pH 5.0	Filter paper discs	Measuring reducing sugars by DNS method	Eveleigh *et al.,* 4
Xylanase (Xyl)	0.5 mL acetate buffer of pH 5.0	Xylan	Measuring reducing sugars by DNS method	Wood and Goodenough, 21
Laccase (Lac)	0.1 M phosphate buffer of pH 6.0	ABTS	Change in absorbance measured at 590 nm	Bourbonnais and Paice, 1
Manganese peroxidase (MnP)	0.5 M Sodium tartarate at pH 5	1mM Guaiacol 1mM $MnSO_4$ & 1mM H_2O_2 (Co-substrates)	Oxidation of Guaiacol measured at 465 nm	Mata and Savoie, 10
Versatile peroxidase (VP)	Tartaric acid at pH 6.5	0.1mM Reactive black dye	Decrease in absorbance due to dye decolourization	Ruiz-Duenas *et al.,* 16

of the substrate. Five randomly selected unopened fruiting bodies of each strain were selected and the morphometric observations viz., individual fruit body weight, pileus thickness, pileus diameter and stalk length were recorded.

The experiment was conducted in completely randomized block division (CRBD) with three replications and three blocks for each replication. Two consecutive trials were conducted to validate the yield data. An analysis of variance was conducted for all variables and a comparison of means was done according to Duncan's test using R-Studio software (version 1.0.136).

RESULTS AND DISCUSSION

The radial growth rate of different strains on WEA medium varied between 0.40 to 0.42 cm/day and the linear growth rate ranged between 0.38 to 0.45 cm/day on un supplemented and 0.41 to 0.59 cm/day on supplemented wheat straw (Table 2). The radial and linear growth rate of different strains on WEA medium and on straw without supplementation was found non-significant. However, linear growth rate of different strains varied significantly on supplemented wheat straw. Addition of wheat bran as a source of nitrogen to the straw significantly increased the mycelial growth rate of all the strains compared to the un supplemented straw. The results are justifying the stimulation effect of available nitrogen on vegetative growth of fungal strains. The highest growth rate was recorded in strain no. DMRO-327 (0.59 cm/day) followed by DMRO-34 (0.56 cm/day) and DMRO-412 (0.54 cm/day). Moonmoon *et al.,* 13 and Kapoor *et al.,* 8 also reported the increased mycelial extension rate of shiitake strains by the addition of organic supplements to the substrate.

Table 2. Mycelial growth rate of different strains of shiitake (radial growth on WEA medium at 10 days of inoculation, linear growth after 21 days of inoculation).

Strain	Radial growth on WEA medium (cm / day)	Linear growth on un supplemented straw (cm/ day)	Linear growth on supplemented straw (cm/ day)
DMRO -34	0.42 ±0.00	0.40±0.04	0.56±0.01
DMRO -35	0.41 ±0.01	0.38±0.03	0.41±0.03
DMRO -51	0.42 ±0.01	0.42±0.01	0.49±0.01
DMRO -297	0.42±0.01	0.41±0.02	0.49±0.02
DMRO -327	0.42±0.01	0.45±0.01	0.59±0.02
DMRO -328	0.40±0.00	0.38±0.01	0.42±0.01
DMRO -330	0.42±0.00	0.41±0.01	0.45±0.01
DMRO -410	0.41±0.01	0.39±0.01	0.47±0.01
DMRO -412	0.41±0.00	0.45±0.03	0.54±0.03
CD	NS	NS	0.054
SE(m)	0.006	0.018	0.018

Means ± standard deviation for three replicates

As the risk of contamination is high at the early growth stages, reduction in time required for complete mycelial colonization is of prime importance while choosing a specific strain for cultivation on straw. Mata *et al.,* (11) reported that the speed of initial mycelial colonization expedites the further utilization of the substrate during the process of solid state fermentation and decides the adaptability of specific strain to the given substrate. The correlations tested in the present study between the linear growth rate on enriched straw and BE of different strains was found significant with the r value of 0.752. This positive relation found between speed of growth rate and yield potentiality supports the above inference. Interestingly, the strains with quick colonization rate took more no. of days for fructification compared to slow growing strains. This may be due to the maximum resource utilization by quick growing strains by secreting the hydrolytic enzymes for a longer period in the incubation phase and in turn to express the optimum yield potentiality. In light of the present data, it is considered that study of growth rate on enriched wheat straw is a good indication to establish the strain-substrate compatibility.

L. edodes being a white rot fungus produces wide range of oxidases and hydrolases for degradation and utilization of various lignocellulosic wastes (Sharma *et al.,* 18). The study of variations in these extra cellular enzyme activities in individual strains at different growth stages gives an insight into the role of enzymes in bioconversion of the substrate. Despite of quantitative variation in individual strains, the pattern of enzyme secretion into the substrate followed the similar fashion in all the strains. The activities of CMCase (Fig.1), FPase (Fig.2) and xylanase (Fig.3) were found highest at the time of primordial formation.

The activity of oxidase enzymes such as laccase (Fig.4) and MnP (Fig.5.) were found highest at 7 days of spawning and later the enzyme activity declined. The activity of VP was increased till the complete colonization of substrate and reduced thereafter (Fig.6). As reported by Crestini *et al*., 2, the availability of alkaline lignins in the straw cell wall acted as inducers for laccase and MnP at initial growth stages and resulted in highest activity. But laccase activity followed the descending trend from spawn run stage to fruiting stage in all the strains. Similarly, the activity of MnP was untraceable, once the mycelium clearly established in the substrate. The

pattern of lesser laccase activity and inactivation of MnP with the progression of mycelial growth is due to the fact that, lesser lignin in straw cell wall available for oxidation by these enzymes (Janusz *et al.,* 6).

Among the activity of cellulases, CMCase was found highest followed by FPase and Xylanse at the time of fruiting. In the present study, CMCase, FPase and xylanase activities of all the strains were followed two different peaks, one at the initial stages of spawn run and another peak at the time of primordial formation. The peak at initial stages of spawn run may be due to the activities present in the spawning material and then the activities gradually reached a plateau with the progression of colonization. The second peak was corresponded with the primordial formation stage. This raise in enzyme activity at fruiting is an indication of ability of the strains to utilize the water soluble carbohydrates for fruit body formation. Similar raise in cellulases activity at the time fruiting of *L. edodes* strains on wheat straw was reported by Mata and Savoie, 10. The role of lignolytic enzymes in substrate utilization and fruiting of shiitake on wood based substrates were specified in many studies (Silva *et al.,* 20). But validating those results on straw based substrate is inappropriate, as the availability of hydrolysable cellulose and non-cellulose polysaccahrides varies with the straw cell walls compared to the wood particles where these compounds are bounded by lignin (Sanchez, 17). Even though, the correlation between the enzyme activities and biological efficiency of different strains were found non significant, the activity of cellulases was significantly greater in high yielding strains than in the strains with low yields. The activity of CMCase and xylanase were significantly highest in strain no DMRO-327 which was also recorded highest BE of 60.23%. These results are clearly indicating the role of cellulases activities at fruiting and in turn increasing the productivity of shiitake in cellulose rich substrates.

The data recorded from two continuous cultivation trials on productivity of different strains of shiitake on wheat straw is presented in Table 3. For moderate productivity of shiitake strains, it requires more than three months pre harvest period on saw dust. But, the average time recorded for first harvest on straw based substrate is lesser than 88 days. In the present study the data clearly showed the reduction in time required for completion of cropping cycle by growing shiitake on enriched wheat straw. This faster growth and early fruiting in straw based substrate is because of the presence of higher amounts of easily available cellulose and hemicelluloses compounds in the straw cell wall compared to the wood substrate (Philippoussis *et al.,* 14).

In both the trials, only one flush was harvested in all the strains and no further yield response was noticed with the subsequent cold shock treatments. The fresh mushroom yields of different strains ranged from 37.84 g to 202.32 g per one kg of wet substrate. The highest yield was recorded with DMRO- 327 with a pooled mean yield value of 202.32 g per kg of wet substrate followed by DMRO-34 (130.70g) and

Table 3. Productivity of different strains of shiitake on wheat straw based substrate.

Strain	IP (No. of days)	Days for first harvest	Total yield (g)			BE (%)	PP	PR
			Trial -1	Trial-2	Mean			
DMRO -34	92.00	99.33	150.58 ±37.25	130.70 ±11.22	140.64±24.20 ab	41.88±7.24 ab	103.33	0.44±0.11 ab
DMRO -35	70.66	83.33	32.37 ±6.97	43.32 ±7.21	37.84±7.07 b	11.24±2.10 b	101.33	0.10±0.02 b
DMRO -51	76.33	88.00	44.03 ±6.12	49.08 ±8.66	46.56±1.86 b	13.84±0.53 b	92.00	0.14±0.02 b
DMRO -297	66.33	75.33	40.25 ±4.55	50.22 ±15.70	45.24±5.80 b	13.44±1.69 b	79.33	0.15±0.02 b
DMRO -327	84.00	91.17	211.10 ±11.38	193.54 ±13.98	202.32±12.59 a	60.23±3.74 a	95.17	0.67±0.03 a
DMRO -328	89.67	98.00	81.94 ±16.56	74.46 ±18.89	78.20±15.99 ab	23.28±4.75 ab	102.00	0.24±0.05 ab
DMRO -330	74.66	82.50	34.10 ±3.86	66.30 ±22.65	50.20±12.97 b	14.88±3.82b	96.00	0.11±0.01 b
DMRO -410	67.33	76.67	71.02 ±5.30	115.81 ±43.69	93.42±19.25 ab	27.72±5.64 ab	99.00	0.22±0.02 ab
DMRO -412	87.00	95.00	152.75 ±18.87	127.87 ±24.44	140.31±20.79 ab	41.79±6.17 ab	80.67	0.57±0.07 ab
CD	7.72	5.06	27.00	38.36	27.88	8.27	4.15	0.09
SE(m)	2.55	1.67	8.93	12.68	9.22	2.74	1.37	0.03

Means ± standard deviation for three replicates, when followed by the same letters, the means were not significantly different (p=0.05)
IP -Incubation Period (time needed for primordial appearance)
PP- Production Period (time from spawning to the last harvest of fruit bodies)
PR-Production Rate (BE/no. of days from spawning to last harvest)

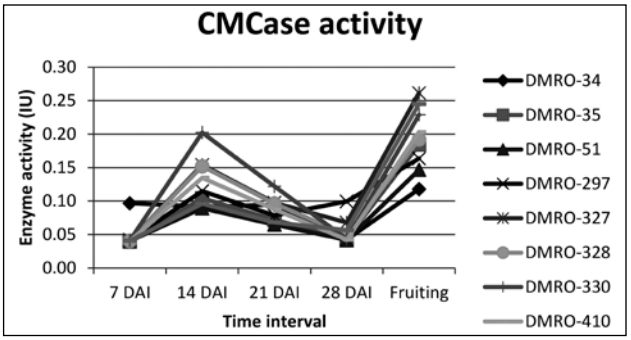

Fig. 1. CMCase activity of shiitake strains at different growth stages

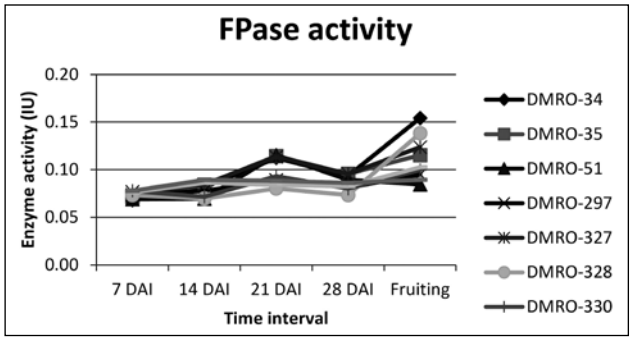

Fig. 2. FPase activity of shiitake strains at different growth stages

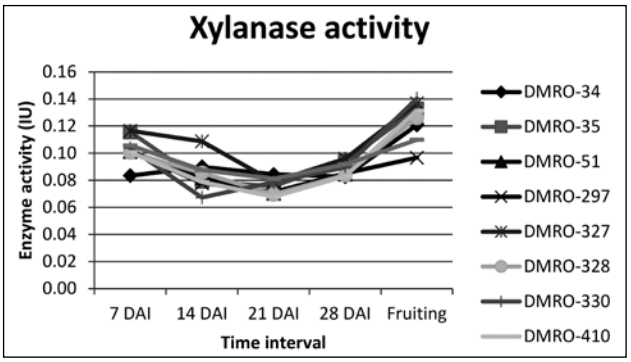

Fig. 3. Xylanase activity of shiitake strains at different growth stages

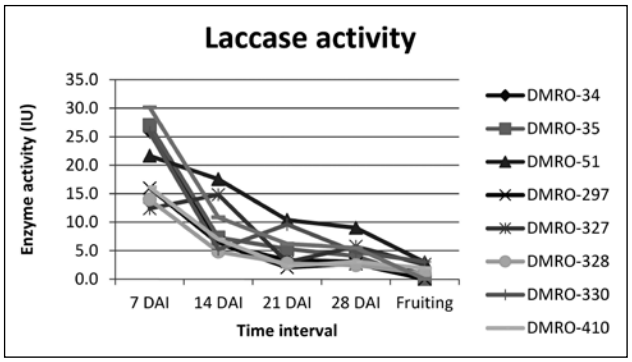

Fig. 4. Laccase activity of shiitake strains at different growth stages

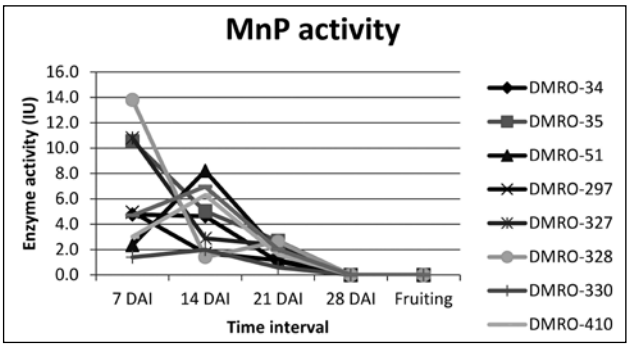

Fig. 5. MnP activity of shiitake strains at different growth stages

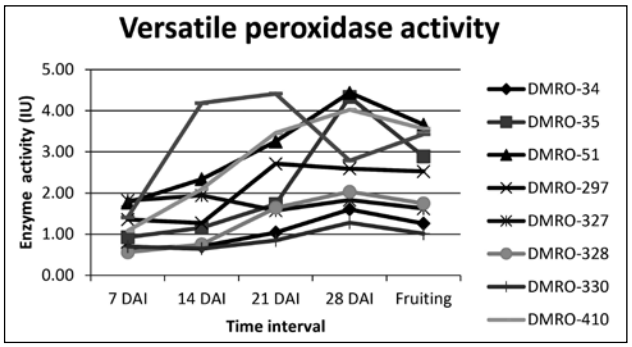

Fig. 6. Versatile peroxidase activity of shiitake strains at different growth stages

DMRO-412 (140.31g). Based on the total biological yield, the strains were grouped into three categories by using the Duncan's comparison test. DMRO-327 was grouped as high yielding strain and DMRO-34, 412, 410, 328 were grouped as strains with medium yield potential and DMRO-35, 51, 297, 330 grouped as low yielding strains. The strain no DMRO-327 was showed BE of 60.23% with the production rate of 0.67. The strains with medium yield potentiality expressed the average BE of 33.67% with the production rate of 0.37. The mean BE of strains grouped under low yield potential was 13.35% with a production rate of < 0.13 (Table 3).

By considering the speed of initial colonization and BE of the strain no. DMRO-327, 34 and 412, they were further selected to grow on pasteurized substrate in block technology. The production kinetics also interprets the relation between incubation period and yield levels. The strains categorized as low yield potential, took an average period of 82.29 days for

first harvest, whereas high and medium yielding strains took an average period of 92 days for first harvest. From the present data it can be inferred that, longer incubation period is required for better productivity in high yielding strains. These significant variations among the strains illustrate the specific relation between genotype and substrate.

The yield attributing factors such as average fruit body weight and thickness of pilues are varying significantly among different strains (Table 4). Strain no DMRO-327 was showed significantly maximum fruit body weight (48.58g) followed by DMRO-328 (38.21g). The thickness of the pileus which is the essential physical quality parameter for drying and fresh market was found highest in DMRO-327(16.33mm) followed by strain no DMRO-328 (15.33mm) and DMRO-51(14.33mm). Based on the thickness of pileus the strains were segregated into three grades such as G_1 > 15 mm thickness, G_2 – 10 to 15 mm and G_3 - <10 mm thickness.

This grouping of strains based on quality attributes further helps in the breeding work to develop strains adapted to straw based substrate with ideal quality. The fruit bodies from strain no DMRO-327 and 328 were categorized under G_1. The yield levels of DMRO-412 are comparatively good but the quality of the fruit bodies is inferior and graded in the scale of G_3. The results pertaining to the pileus diameter of different strains were found non significant. However, the study on correlation between the stalk length to pileus diameter ratio and average fruit body weight showed that higher the stipe length lesser the weight of fruit body.

The present investigation provided data useful for screening of available germplasm to grow on straw based substrate. The significant variations among the different genotypes in terms of enzyme activity and yield potential define the specific adaptability of strains towards the straw based substrate and validate the object of present study.

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Table 4. Fruit body quality parameters of different strains of shiitake grown on wheat straw based substrate.

Strain	Average fruit body weight (g)	Pileus thickness (mm)	Pileus diameter (cm)	Stipe length (cm)
DMRO -34	25.35±5.86	13.00±0.57	8.73±1.01	6.13±1.00
DMRO -35	21.90±4.06	12.67±0.88	7.37±0.21	5.07±1.07
DMRO -51	21.61±7.23	14.33±1.86	8.23±0.51	3.70±0.10
DMRO -297	19.66±3.49	13.67±0.33	7.57±0.55	3.40±0.35
DMRO -327	48.58±16.58	16.33±0.882	9.70±1.42	5.93±0.46
DMRO -328	38.21±16.67	15.33±0.58	8.67±2.24	5.70±0.78
DMRO -330	23.34±5.70	12.33±1.20	7.97±0.49	4.97±0.21
DMRO -410	23.15±9.39	11.83±0.44	8.80±1.74	4.60±0.10
DMRO -412	20.78±0.79	9.50±0.29	8.93±2.69	4.37±1.25
CD	17.24	2.68	NS	1.24
SE(m)	5.70	0.89	0.87	0.41

Means ± standard deviation for three replicates.

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