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Full Length Research Paper

Occurrence of maize yellow mosaic virus and evidence of co-infection with maize lethal necrosis viruses in Bomet County, Kenya

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Maize Lethal Necrosis (MLN) disease is caused by synergistic interaction between maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV). However, some East African countries have detected maize infecting polerovirus named maize yellow mosaic virus (MaYMV) also known as maize yellow dwarf virus (MYDV-RMV) co-infecting maize with MLN viruses. Maize yellow dwarf virus occurrence and distribution in different parts of Kenya are not yet elucidated. This study aimed to establish the occurrence of MaYMV in maize and sorghum in Bomet County, MLN hotspot region in Kenya. Maize (n=90) and sorghum (n=19) samples were collected from East and Central sub-counties of Bomet County in 2019/2020. Reverse transcription-polymerase chain reaction (RT-PCR) protocol was developed and optimized for screening the samples using specific primers. Amplicons of 600, 250 and 169 bp were generated for MaYMV, MCMV and SCMV, respectively. The analysis revealed 56% (62/109) of the samples tested positive for MaYMV co-infecting maize with MLN viruses. Sanger sequencing of representative samples confirmed the presence of MaYMV. BLASTN analysis showed 95-100% sequence identity to MaYMV/MYDV-RMV hence confirmed the occurrence of MaYMV infecting maize and sorghum in Bomet County whose impacts is a potential threat to food security.

Key words: Occurrence, co-infection, maize lethal necrosis, maize yellow mosaic virus, maize chlorotic mottle virus, sugarcane mosaic virus.

INTRODUCTION

Maize (*Zea mays* L.) and Sorghum (*Sorghum bicolor* L. Moench) are important cereals crops essential for livelihood and food security in Kenya (De Groote et al., 2016; Njagi et al., 2019). The former can flourish in a wide range of climatic conditions; therefore, it can be cultivated extensively throughout the country (Mwathi et al., 2016). The latter is drought tolerant and mainly grown in arid and semi-arid parts of Kenya, including Western,

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Eastern, North Rift valley, and some parts of the Central province of Kenya (Kagwiria et al., 2019). They are primarily grown for their grains and are closely related in their utilization (One Acre Fund, 2020). For example, they can be milled to flour to make 'ugali' and porridge, typical Kenyan meals. Further, maize and sorghum grains have other uses. They can be brewed into alcoholic beverages, boiled and consumed like rice, baked like wheat, and popped like popcorn for snack (Esilaba et al., 2019; One Acre Fund, 2020).

Maize serves as a leading staple food and source of income and livelihood for most rural farmers in Kenya (Naseem et al., 2018). However, the emergence of Maize Lethal Necrosis (MLN) disease affected maize production making sorghum the second preferred alternative cereal after maize (One Acre Fund, 2020). In East Africa MLN disease is caused by synergistic interaction of MCMV and SCMV (Kiruwa et al., 2016). However, recent MLN studies identified a novel Polerovirus known as MaYMV/MYDV-RMV infecting maize and sorghum in mixed infection with the MLN causing viruses (Massawe et al., 2018; Yahaya et al., 2019).

Maize yellow mosaic virus (MaYMV) belongs to the genus *Polerovirus* (Chen et al., 2016). Together with *Luteovirus* and *Enamovirus* genera; they are members of the *Luteoviridae* family (Garcia-Ruiz et al., 2020). Maize yellow mosaic virus was first reported in China and later in South America and Africa (Chen et al., 2016; Bernreiter et al., 2017; Palanga et al., 2017; Yahaya et al., 2019). Empirical evidence associated MaYMV with yellow mosaic and leaf reddening symptoms with an estimated yield loss of 10-30% in maize (Stewart et al., 2020; Bernreiter et al., 2017). Maize yellow mosaic virus is phloem limited (Garcia-Ruiz et al., 2020). It is efficiently transmitted by corn leaf aphid *Rhopalosiphum maidis* and partially transmitted by *Rhopalosiphum padi (R.padi)* (Stewart et al., 2020). Its genome comprises linear, monopartite, positive-sense single-stranded RNAs, which is approximately 5.3-5.7 kb (Chen et al., 2016). Additionally, the genome has six open reading frames (ORF) encoding protein P0-P5 with three untranslated regions (UTRs), including the 51 UTR, the 31 UTR, and the intergenic UTR between ORF2 and ORF3. Typical to other polerovirus, P0 protein of MaYMV is a potent silencing suppressor (Holste, 2020).

Poleroviruses are likely to interact among themselves or with other viruses from different families. Some of these associations include synergistic interaction between Beet western yellows virus (BWYV) a *Polerovirus* with Beet mosaic virus (BtMV) a *Potyvirus* which leads to fast systemic infection with early and severe symptoms development (Garcia-Ruiz et al., 2020). Similar results were demonstrated by Stewart and Willie (2021) who reported stunting symptoms in MLN triple infection (MCMV+SCMV+MaYMV) which further progressed to MLN disease. Besides, farmers in Kenya plant maize and sorghum in traditional farming system or in the nearby field (Demissie et al., 2020). This practice allows the horizontal spread of common pathogens infecting the two crops such as MCMV, SCMV and MaYMV. Thus, acting as an inoculum for the new non-infected plants especially in Bomet County where farmers practice continuous maize cropping.

Despite the detection of MaYMV in mixed infection with MLN viruses, most studies in Kenya continue to focus on understanding MCMV and SCMV interaction in association with MLN without any consideration of MaYMV co-infection, a potential threat to food security. Therefore, this study sought to establish the occurrence of MaYMV co-infection with MLN viruses in Bomet County, an MLN hotspot region in Kenya.

MATERIALS AND METHODS

Study area

Bomet County (Figure 1) was selected for this study because it is the epicentre of MLN infections. Furthermore, it is classified among the MLN hotspot regions in Kenya (Wangai et al., 2012). The County lies between latitudes 0° 29' and 1° 03' south and between longitudes 35° 05' and 35° 35' east (County Government of Bomet, 2018). Maize yellow mosaic virus incidence in Kenya remained to be established. However, previous MLN studies identified MaYMV in mixed infection with MLN causing viruses suggesting potential with high distribution (Massawe et al., 2018; Mwatuni et al., 2020).

Sample collection and RNA extraction

Maize and sorghum samples were collected from East and Central Sub-Counties of Bomet County in 2019/2020 (Figure 1). In total, 90 symptomatic maize (Figure 2a) and 19 asymptomatic sorghum (Figure 2b) samples were collected from the visited farmers' fields.

A handheld Global Positioning System (GPS) was used to record the coordinates at the samples collection points. A zig-zag pattern was adopted during sampling in the farmers' fields. A polythene bag was inverted over one hand and used to grip a portion of the leaf to be sampled. The other hand was used to cut the leaf off into the inverted polythene bag while maintaining the leaf sample inside the pack following a procedure described by Mezzalama et al. (2015). The samples were labelled and placed in a cool box containing dry ice and transported to Kenya Agricultural and Livestock Organization (KALRO) Biotechnology laboratory and stored at -80°C.

In the laboratory, total RNA was extracted from 0.1 g of the collected maize and sorghum samples using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturers' instructions.

Primer design and validation

Maize yellow mosaic virus primers were designed using primer3 software package. A Kenyan isolate with the accession number (MH205607.1) was used as a reference sequence. Eleven overlapping primers were generated spanning the entire reference genome (Table S1). To validate the primers efficiency, Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using MaYMV positive RNA sample previously used for generation of complete sequence of MYDV-RMV (MH205607.1) with the eleven overlapping primers.

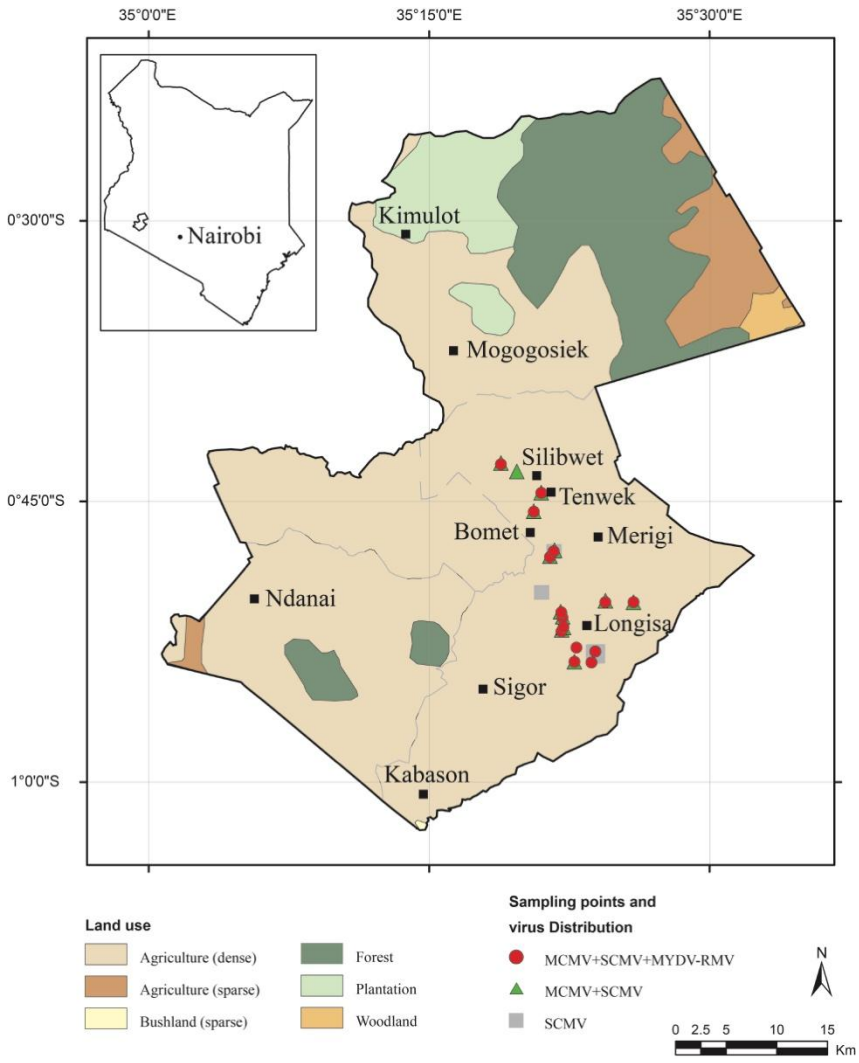

Figure 1. A map showing the main land use in Bomet county, sampling points and virus distribution in Bomet East and Central sub-counties.

Reverse transcription polymerase chain reaction optimization and detection

Two step RT-PCR was optimized using specific primers (MYDV-RMV_1, MCMV_1 and SCMV_1) and representative symptomatic maize samples from Bomet county. Reverse transcription was carried out using Thermo Scientific RevertAid First Strand cDNA Synthesis kit following manufacturers' instructions. Briefly, RNA (2 µl) was used as a template. A 12 µl reaction volume containing 1 µl of Oligo primer, and 9 µl of nuclease-free water was incubated in hot water bath at 65°C for 5 min then chilled immediately on ice. Reverse transcription (RT) was done in 20 µl reaction volume at 45°C for 60 min, followed by reaction termination at 70°C for 5 min.

PCR amplification was carried out using Thermo Fisher Scientific DreamTaq PCR Master Mix (2X) which contained DreamTaq DNA Polymerase, 2X DreamTaq buffer, dNTPs, and 4 mM $MgCl_2$ following manufacturers' instructions. Thirty-five cycles of PCR amplification were completed in Veriti™ 96-Well Thermal Cycler (The Applied Biosystems™, Carlsbad, CA, USA). Typically, the PCR program begun with DNA initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 30 s, primer annealing between 52.8 -60°C (based on the specific primers) for 30 s, extension at 72°C for 1.5 min, and end with a final extension at 72°C for 5 min. The PCR products were analysed by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The amplicons were viewed under a UV transilluminator (UVTEC Essentail V6 from UVItec.Ltd Cambridge).

Sanger sequencing and sequence analysis

The PCR products (4 µl) from 19 representative samples were enzymatically cleaned using 10 µl of ExoSAP™ (Thermo Fisher, Waltham, USA) following the manufacturer's instructions. Briefly, enzymatic cleaning was carried out in Veriti thermocycler (Applied Biosystems, Carlsbad, CA, USA) at 37°C for 15 minand 80°C for 15 min. BigDye™ Terminator Sequencing Kit (Thermo fisher Scientific) was used to perform cycle sequencing using the Sanger Method. Base-calling was performed upon completion of the analysis, AB1 file was generated ready for bioinformatic analysis.

The sequenced data was edited for quality using ChromasPro

Figure 2. A typical maize and sorghum samples collected from farmers' fields. (a) shows symptomatic maize plant displaying leaf chlorosis and leaf blade necrosis and (b) shows asymptomatic sorghum.

software (Technelysium Pty Ltd, South Brisbane, Australia). Consensus sequences were generated using CAP3 software (Huang and Madan, 1999). BLASTN analysis was used to identify the close relatives of MaYMV. Multiple sequence alignments of the 19 nucleotide sequences from this study and twelve MaYMV nucleotide sequences from NCBI GenBank database were done using CLUSTALW software (Thompson et al., 2002). The downloaded nucleotide sequences included Nigerian isolate (KY684356.1), Ecuador isolate (KY052793.1), Brazil isolate (KY940544.1), China isolates (KU291103.1, KU291100.1, KY378940.1, and KT992824.1), Tanzanian isolate (MG664794.1), Ethiopian isolate (MF684368.1), South Africa isolate (MG570476.1), Kenya isolates (MF974579.2 and MH205607.1) and Cassava brown streak virus (KR911746.1) was used as an outgroup. A phylogenetic tree showing evolutionary relationship was generated using unweighted pair group method with arithmetic mean (UPGMA) method with 1,000 bootstrap replication using Molecular Evolutionary Genetics Analysis (MEGA) version 6 software package (Tamura et al., 2013).

RESULTS AND DISCUSSION

Primer design and validation

Primer design led to generation of eleven overlapping primers for MaYMV (Table S2). Validation of the primers amplified expected 600 bp for all the designed primers as shown in Figure 3a. This revealed the efficiency of all the designed primers for molecular detection of MaYMV. Hence, they can be used for RT-PCR detection and Sanger Sequencing of MaYMV.

Reverse transcription polymerase chain reaction optimization and virus detection

The results of RT-PCR optimization amplified 600, 250 and 169 bp for MaYMV, MCMV and SCMV, respectively (Figure 3b). This amplification demonstrates the competency of RT-PCR protocol and the selected specific primers, (MYDV-RMV_1, MCMV_1 and SCMV_1), for detection of MaYMV, MCMV and SCMV, respectively.

The RT-PCR detection results (Table 1) revealed double infection of MCMV and SCMV in 19% or (20/109) of the analysed samples. Similar double infection was responsible for maize yield losses of 59% or 300,000 tons in moist transitional zones, mainly in Western Kenya (De Groote et al., 2016).

Maize lethal necrosis disease is caused by combined infection of Maize chlorotic mottle virus (MCMV) with any member of genus potyvirus such as Sugarcane mosaic virus (SCMV), Maize dwarf mosaic virus (MDMV) or Wheat streak mosaic virus (WSMV) (Isabirye and Rwomushana, 2016). In South Rift region of Kenya, MLN was confirmed to be caused by synergistic interaction of

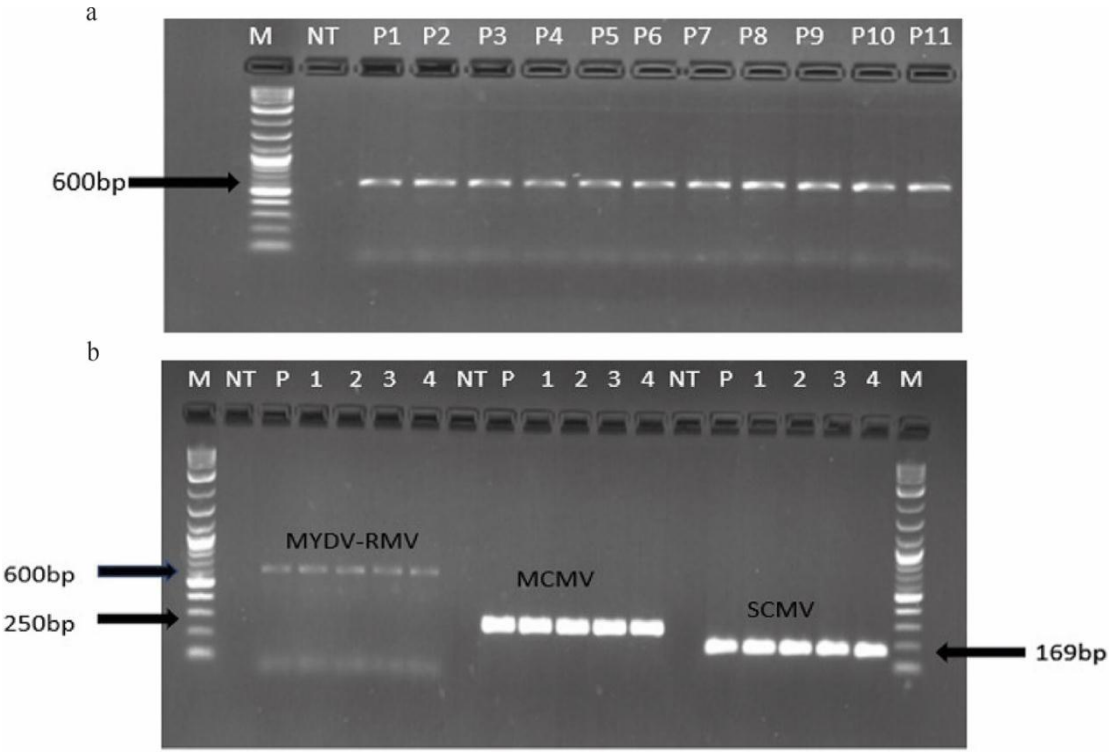

Figure 3. Validation of MYDV-RMV primers and optimization of RT-PCR detection method. **(a)** Amplicon of 600 bp for MaYMV. Lane M represent DNA ladder; lane NT represent a negative control and lane P1-P11 are the 11 primer pairs. (**b)** RT-PCR amplification of 600 bp, 250 bp and 169 bp for MYDV-RMV, MCMV and SCMV, respectively. Lane M is DNA ladder; lane NT is negative control, lane P-positive control and lane 1-4 are representative samples co-infected by the three viruses.

Table 1. Number of MaYMV, MCMV and SCMV positive samples in single and mixed infection from maize and sorghum leaves collected in Bomet county.

Sub-County	Host	MCMV+SCMV+ MaYMV	MCMV+SCMV	SCMV	Negative samples	Total
Bomet East	Maize	32	11	3	4	50
	Sorghum	0	0	5	6	11
Bomet Central	Maize	30	8	1	1	40
	Sorghum	0	1	0	7	8
Total		62 (56%)	20 (19%)	9 (8%)	18 (17%)	109

MCMV and SCMV (Leitich et al., 2020). The results showed the continued occurrence of the MLN disease in Bomet County.

This study further revealed the occurrence of MaYMV in 56% or (62/109) of the analysed samples (Table 1). It is worth noting that MaYMV was only detected in mixed infection with MLN causing viruses, MCMV and SCMV. These results concurred with those reported by Massawe et al. (2018) who also detected MaYMV in mixed infection with MCMV and SCMV in maize. Besides, the triple infection (MCMV+ SCMV+ MaYMV) was present in all the 18 maize fields where 90 symptomatic maize samples were collected This indicated the occurrence of MaYMV in Bomet County in mixed infection with MCMV and SCMV the MLN causing viruses.

The detection rate of triple infection (MCMV+ SCMV+ MaYMV) was higher (62/109) than the detection rate of the double infection (MCMV+SCMV) of MLN causing viruses (20/109) (Table 1). This is indicative of high occurrence of MaYMV in Bomet County as shown in Figure 1, which may be a potential threat to food security. Furthermore the synergistic interaction between MaYMV and the MLN causing viruses (MCMV+SCMV+MaYMV) enhance stunting in maize which further progressed to MLN disease despite suppression of increased MCMV titer induced by SCMV in double infection (Stewart and

Willie, 2021). Thus, presenting unknown potential disease impact of MaYMV in single and in mixed infection.

SCMV was the most abundant virus in the study site. It was detected in triple and double infection and 8% (9/109) samples were positive for its single infection. Thus, it was confirmed that SCMV was the major potyvirus causing MLN in South Rift region of Kenya as reported by Leitich et al. (2020). All the sorghum samples tested negative for MaYMV by RT-PCR. However, representative Sanger Sequenced sorghum samples were positive. This might be associated with low sensitivity of RT-PCR as compared to Sanger Sequencing which is able to detect low viral concentration. Besides MaYMV are restricted to the phloem (Garcia-Ruiz et al., 2020), hence the viral concentration on the leaf tissue could be low.

The low MLN incidence on sorghum observed in this study could be attributed to the tolerance nature of sorghum to MLN viruses which results in low viral titer level. Plant cultivars play a crucial role in disease symptoms expression and viral titer concentration during plant development for example MLN susceptible maize hybrid developed severe MLN symptoms coupled with increased viral titer concentration at an early stage of development as compared to less susceptible hybrid (Leitich et al., 2021). Besides, all the sorghum collected were landraces (local varieties) which the farmers believe they are resilient and can resist diseases.

Polerovirus are reported to be the most damaging viruses infecting more than 32 monocot and dicot plants in *Luteoviridae* family (Garcia-Ruiz et al., 2020). For instance, the Potato leafroll virus, the first identified *Polerovirus*, is associated with a 50-60% yield loss in potatoes equivalent to 100 million dollars annually in the United States (Holste, 2020). Likewise, MaYMV occurrence may be accountable for the substantial maize losses observed in the farmers' fields in Bomet County.

Synergistic interaction between poleroviruses and other viruses have been reported by Holste (2020). A classical illustration is the interaction between Potato leafroll virus (PLRV) (*Polerovirus*) co-infection with either Potato virus X (PVX) or Potato virus Y (PVY) (*Potyviruses*), resulting in increased symptoms severity and yield loss (Garcia-Ruiz et al., 2020). Similarly, the synergistic interaction of MaYMV with MLN viruses MCMV and SCMV may be responsible for the severe symptoms characterized by bright yellow symptoms on the leaf surface and stunted growth with small or no ear formation of maize as observed in the farmers' fields in Bomet County. Furthermore, the triple infection (MCMV+SCMV+MaYMV) is reported to cause maize stunting which advance to MLN disease (Stewart and Willie, 2021).

Sanger sequencing and sequence analysis

Sanger Sequencing further confirmed the presence of MaYMV in both maize and sorghum (Table S2). BLASTN analysis showed 95-100% identity of our samples to MaYMV/MYDV-RMV with 100% coverage. Similar results were reported by Wamaitha et al. (2018) who also detected MaYMV in mixed infection with MCMV and SCMV using Next Generation Sequence (NGS) in maize and sorghum samples collected in Embu and Kirinyaga County which are low MLN hotspot regions in Kenya.

Phylogenetic analysis of 19 sequences from this study and 12 from NCBI database were separated into four groups based on geographical location named as Africa Isolate (AI), Asian Isolate (ASI) and South America (SA). The 19 isolates from this study exclusively clustered together with isolates from different parts of Africa as shown in Figure 4. This showed close relationship and insignificant variation among the MaYMV African isolates. The same observation was made by Yahaya et al. (2019) who also described geography specificity of MaYMV independent of their host. Interestingly, the African isolates shared a node with the China Isolates. This indicated a potential common ancestral origin. However, the South America isolates were separated displaying some variation between them. Cassava brown streak virus (CBSV) was used as an outgroup.

Conclusion

This study established the occurrence of recently reported maize infecting polerovirus MaYMV/MYDV-RMV in co-infection with MLN causing viruses in Bomet County. The results showed that MaYMV co-infect maize and sorghum with MLN causing viruses MCMV and SCMV and the triple infection of (MCMV+SCMV+MaYMV) is higher 56% compared to the double infection (MCMV+SCMV) which was 19%. Considering the potential losses associated with the MaYMV co-infection, we recommend MaYMV to be integrated in development of MLN control/management strategies and a need to determine the impact associated with MaYMV in single and mixed infection on yield.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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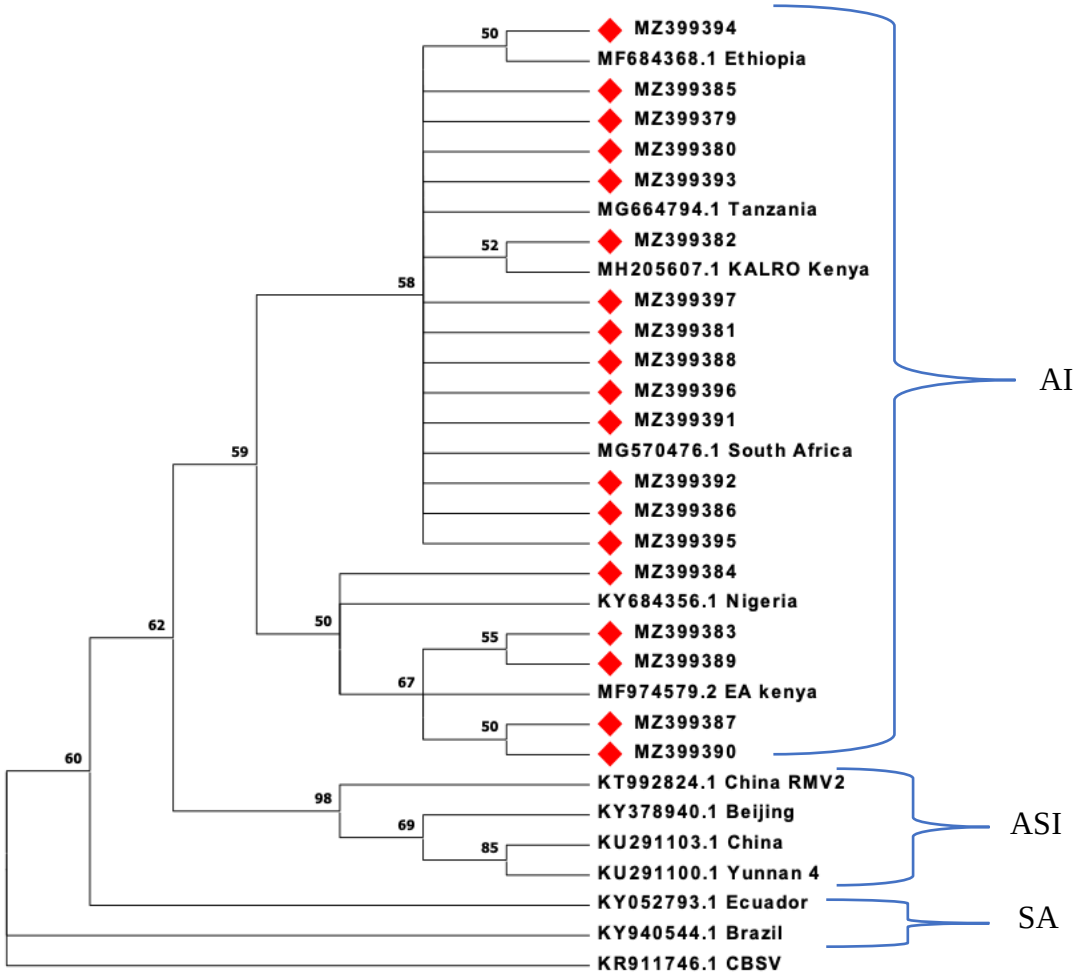

AI-African isolates, **ASI**-Asian isolates, and **SA**-South American isolates.

Figure 4. Phylogenetic tree of partial nucleotide sequence of P1 and P2 proteins showing evolutionary relationship of MaYMV isolates generated from this study marked in red dots (n= 19) with others from NCBI (n=12). Phylogenetic tree was generated by UPGMA method with 1000 bootstraps replicates using MEGA 6 software. Branches with less than 50% bootstrap support have been collapsed. CBSV sequence was used as an outgroup.

designed MaYMV primers. Mary Lechuta and Michael Njoroge for their assistance in laboratory and bioinformatic analysis, respectively. This work was funded by the World Bank through Kenya Climate Smart Agriculture (KCSAP) (Grant Number 20076) and National Research Fund (NRF).

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SUPPLEMENTARY TABLES

Table S1. Primers used for detection and amplification of Maize yellow dwarf virus-RMV (MYDV-RMV), maize chlorotic mottle virus (MCMV) and sugarcane mosaic virus (SCMV).

Primer name	**Forward 5'-3'**	TM (°C)	**Reverse 5'-3'**	**TM** (°C)
MYDV-RMV_1	ACCCAAGGGAGTGCCTAAAG	60.5	TATCGCGACGAGACATGAAC	59.8
MYDV-RMV_2	CCACATCGGGGTTTTATCAG	60.2	GTCCTTGAAAAAGGCTCACG	59.9
MYDV-RMV_3	CTTGTCGCCTCGACTAGGAC	60.0	TCGTTTCCCTGAAACTTTGG	60.1
MYDV-RMV_4	CGACCTCGTCCACTTCAAAT	60.1	GCTCCCTGTCCTCAGTTGAC	59.8
MYDV-RMV_5	CGCAGCTGAACTGAAAAGC	59.9	ACTCTCGCGATTGGTCATCT	59.8
MYDV-RMV_6	CTCATTGCTGGATCAACTGG	59.2	GTGCGAGGTATTCCTTCTCG	59.8
MYDV-RMV_7	TCAGTCGACACGTGCCTAGA	60.6	CCACTTGGTCGTCTTCGTCT	60.3
MYDV-RMV_8	GAACGTGCGTTCAATTGTGA	60.7	CGGGTTTTGAACATTGACCT	60.8
MYDV-RMV_9	ATGCGCCATCCTCTACAAAG	60.2	TTGAGATCAGGGTGTGCTTG	59.8
MYDV-RMV_10	GTTGGCAGGCTTACTCATGG	60.5	CAAACGAACTTGGGAGGATT	59.1
MYDV-RMV_11	GCTGAACCAGCATCGAAAGT	60.4	CTCCCGGAAACCTCTCTCTT	59.8
MCMV_ 1	AACATTCACAGCAGACACC	54.3	GATAGCCACAATGAATCGTCC	59.4
SCMV_ 1	TCTACTGAGCGATACATGCC	56.5	CGTGTGTTTGAACCACGAAC	60.0

Table S2. BLAST search analysis results showing close relative of the sequenced samples.

Sample ID	**Sample Accession no:**	**Close relative**	**Query cover (%)**	**E-value**	**% Identity**	**Accession no:**
BE25	MZ399379	Maize yellow mosaic virus isolate 16/0111 suppressor of RNA silencing	100	0.0	99.81	MG664792.1
BE15	MZ399380	Maize yellow mosaic virus isolate T2F2S5 P3-P5	99	0.0	99.81	MF425875.1
BE18	MZ399381	Maize yellow dwarf virus-RMV isolate KARLO, complete genome	100	0.0	99.62	MH205607.1
BE48	MZ399382	Maize yellow dwarf virus-RMV isolate KARLO, complete genome	100	0.0	99.44	MH205607.1
BE10	MZ399383	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	99.81	MF684368.1
BE3	MZ399384	Maize yellow dwarf virus-RMV isolate KARLO, complete genome	100	0.0	99.81	MH205607.1
BE28	MZ399385	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	99.62	MF684368.1
BC32	MZ399386	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	99.81	MF684368.1
BE36	MZ399387	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	100	MF684368.1
BC19	MZ399388	Maize yellow mosaic virus isolate 16/0092 suppressor of RNA silencing	100	0.0	100	MG664791.1
BC8	MZ399389	Maize yellow mosaic virus isolate T2F3S4 P3-P5	100	0.0	99.43	MF425876.1
BC14	MZ399390	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	100	MF684368.1
BC2	MZ399391	Maize yellow dwarf virus-RMV isolate, KARLO complete genome	100	0.0	99.24	MH205607.1
BC25	MZ399392	Maize yellow mosaic virus isolate T2F2S5 P3-P5	100	0.0	99.62	MF425875.1
BC39	MZ399393	Maize yellow mosaic virus isolate MV90 partial genome	100	0.0	99.81	MF684368.1
SBE39	MZ399394	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	99.61	MF684368.1

Table S2. Contd.

SBE54	MZ399395	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	99.8	MF684368.1
SBE45	MZ399396	Maize yellow mosaic virus isolate LETF2S1 P3-P5	100	0.0	95.57	MF425861.1
SBC16	MZ399397	Maize yellow mosaic virus isolate MV90, partial genome	100	0.0	97	MF684368.1

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Bioconversion of bananiculture waste for Amazon edible mushroom production

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The objective of this study was to evaluate the use of banana stalk and pseudostem residues from two banana cultivars as cultivation substrate for the Amazonian *Pleurotus ostreatus* NATB. The residues of the two cultivars (silver-dwarf and thap-maeo) were used to cultivate *P. ostreatus* NATB from the Mushroom Cultivation Laboratory Micoteca, CTI, INPA, Brazil, reactivated in medium Potato Dextrose Agar, and subsequently incubated in medium composed of banana juice infusion broth. The tertiary matrix or spawn consisted of solid residue without supplementation that was properly autoclaved for secondary matrix fragment incubation. The treatments were performed in HDPE bags with 1 kg of autoclaved residues, and were humidified at 75% with 5% of tertiary culture. Each treatment consisted of 20 repetitions incubated in a chamber at 25ºC for 45 days. Biological efficiency, yield, and loss of organic matter were evaluated. Mushrooms grown on dwarf silver pseudostem substrate had the highest average percentages in efficiency, biological and yield, but the loss of organic matter was not directly related to these productivity parameters or behavior that can be attributed to other factors such as CO_2 and water loss during the process.

Key words: *Pleurotus ostreatus*, agribusiness, biodegradation.

INTRODUCTION

Banana is the second most consumed fruit in the world. It is grown in over 120 countries, and it has a great socioeconomic significance. In addition to being an important human food, it also contributes to the trade balance of many countries as an important export product, generating profit and income for economies. In

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2014, Brazil was the fourth largest producer with 6,953.747 million tons, behind only India, China and the Philippines according to data from FAOSTAT (2014).

As a consequence of large scale banana production, there is abundant waste generation. An estimated 30% of post-harvest value is lost, and this does not consider the waste generated from pseudostem, stalk, straw, and leaves (Carvalho et al., 2011). These residues are considered the most important in terms of volume generated and fibrous potential, but they are usually used as fertilizer in banana plants (Soffner, 2001).

The utilization of banana agroindustry residues can be applied in several value-added products including basketry, cardboard, fibers, and handicraft products because of the resistant fibers. However, they are even more widely applicable, and applications in biotechnological processes seek to discover new products, reduce environmental impacts, and add value to generated products.

Several bioprocesses have been developed using these materials as substrate to produce various high added value molecules like microbial proteins, organic acids, ethanol, enzymes, and biologically active secondary metabolites (Peralta et al., 2008).

Another important bioprocess is the production of edible mushrooms from the reused agro-industry residues. This includes the production of edible mushrooms in banana straw (Bonatti at al., 2003; Bonatti et al., 2004), in cotton waste (Holtz et al., 2009; Gonçalves et al., 2010), peach palm waste (Duprat et al., 2015), palm oil agribusiness waste (Morais et al., 2017), corn silage (Oliveira et al., 2018), and cupuaçu bark (Fonseca et al., 2015) among others.

Among the diversity of edible mushrooms, fungi of the genus *Pleurotus* can grow on a wide variety of agricultural residues. In addition to their protein quality and the presence of essential amino acids, the mushroom *Pleurotus ostreatus* is an important source of carbohydrates, fiber, minerals and vitamins, making it a nutritionally rich food (Rampinelli et al*.,* 2010).

Given the above, this study evaluated the use of different banana cultivar residues as substrate for cultivating the Amazonian edible mushroom *P. ostreatus* NATB.

MATERIALS AND METHODS

Fungal lineage

The strain of the fungus *P. ostreatus* NATB was accessed from the collection of the Edible Fungi Laboratory of the National Institute of Amazonian Research - INPA. Small fragments were aseptically inoculated in Petri dishes in Potato Dextrose Agar (PDA) medium for colony activation.

Waste treatment

The two banana cultivars (silver-dwarf and thap-maeo) and their respective residues (pseudostem and stalk) were collected from small farmers in the municipality of Parintins-Am (Brazil). The primary processing consisted of subjecting the pseudostem and the “in natura” banana stalk to the mechanical action of a trap 200 organic shredder. The residues were deemed shredded after this step. This material was dried outdoors and packed in plastic bags. Crushed pseudostem and stain residues were autoclaved at 121°C for 60 min for asepsis. After cooling, it was used to formulate an alternative culture medium.

Secondary matrix

P. ostreatus NATB inoculum (9 mm diameter discs) previously micellated in BDA medium were transferred to Petri dishes containing alternative culture medium prepared from infusion of banana residues (pseudostem and stalk) to obtain the secondary matrix, which served as a source of inoculum for the seed in culture medium made from the residue of the thap-maeo cultivar (PSTM), dwarf silver-pseudo-stem (PSPA), thap-maeo cultivar (ENTM) and the stalk of the silver dwarf cultivar (ENPA).

Culture media were prepared by infusing 100 g of substrate in 1 L of boiling water for 30 min, filtering on cotton and making up to 1.5 L. It was necessary to add 2% $CaCO_3$ to adjust the pH (6.5) in 98% residue. After filtration, 12 g of dextrose and 15 g of agar were added to each medium. The different media were autoclaved at 121°C for 60 min and then in a fully stereo environment were poured into Petri dishes, incubated at 25ºC in a BOD chamber.

Tertiary Matrix "Spawn"

The matrix was elaborated from the adapted methodology of Eira and Minhonhi (1997) and Sales-Campos (2008). The substrates were homogenized and humidified at 75% and then deposited in 500 ml glass vials in the amount of 500 g which were drilled in the central portion for secondary matrix inoculum packaging, closed and autoclaved at 121°C for 60 min. After cooling under sterile conditions, mycelium fragments of the secondary matrix were inoculated in the glass vials according to the prepared substrates (PSPA, PSTM, ENPA, ENTM). The flasks were closed and kept in BOD at 25ºC until the complete substrate colonization by the fungus. This matrix served as inoculation source for the cultivation substrates for *P. ostreatus* mushroom production of the present study.

P. ostreatus NATB cultivation

The substrates for fungal growth were prepared using dry and crushed residues (pseudostem and stalk) and four treatments (PSPA, PSTM, ENPA, ENTM) with twenty repetitions for each treatment. The humidity was set to 75%. $CaCO_3$ was not added because the mixture pH did not require correction. The substrates were inserted into HDPE (high density polyethylene) bags, drilled into the central portion for tertiary matrix inoculation, and autoclaved for 60 min. After cooling of the bags, portions removed from the tertiary matrix were inoculated in a laminar flow chamber in the substrate bags. The bags received a synthetic sponge breather to facilitate gas exchange, and they were randomly placed in a chamber with controlled temperature and humidity at 80% humidity and 25ºC (Table 1). The treatments were incubated in the dark for myceliation.

After complete myceliation, the bags were transferred to a production chamber with 90% humidity at 22°C with a 12 h photoperiod to inducing mushroom primordia and basidioma production.

Table 1. Cultivation conditions of *P. ostreatus* NATB in banana substrates.

Cultivate	Substrate	Myceliation humidity (%)	Cultivation humidity (%)	Miceliation temperature (ºC)	Cultivation temperature (ºC)
Prata-anã	PSPA	80	90	25	22
	ENPA	80	90	25	22
Thap-maeo	PSTM	80	90	25	22
	ENTM	80	90	25	22

PSPA: Dwarf silver pseudostem; ENPA: Dwarf silver stalk; PSTM: Thap-maeo pseudostem; ENTM: Thap-maeo stalk.

Table 2. Parameters analyzed during *P. ostreatus* NATB production in substrates of banana cultivars.

Cultivate	Prata-anã		Thap-maeo	
Substrates	**PSPA**	**ENPA**	**PSTM**	**ENTM**
Micellalization (days)	20	27	21	28
Formation of primodia (days)	22	29	23	30
Total culture time (days)	45	45	45	45

PSPA: Dwarf silver pseudostem; ENPA: dwarf silver stalk; PSTM: thap-maeo pseudostem; ENTM: thap-maeo stalk.

Mushroom yield was expressed by calculating biological efficiency during cultivation. Biological efficiency (EB) represents the percentage conversion of substrate to fungal biomass (mushrooms).

$$\text{BE}\ (\%) = \frac{\text{Fresh pasta with mushrooms (g)}}{\text{Dry substrate mass (g)}} \times 100$$

Organic matter loss (OML) is the index that evaluates the decomposition of the substrate by the fungus. This index is based on the loss of fungal decomposed organic matter, which is determined by the difference between the initial substrate dry mass and the residual substrate dry mass. The OML was evaluated according to Sturion (1994), expressed by the following formula:

$$\text{OML}\ (\%) = \frac{\text{Residual substrate dry mass (g)}}{\text{Dry mass of the starting substrate (g)}} \times 100$$

The yield (g/kg) of *P. ostreatus* was calculated using the fresh mass of mushroom produced by the fresh substrate mass used.

$$\text{R}\ (\%) = \frac{\text{Fresh mushroom pasta produced (g)}}{\text{Fresh substrate initial mass (kg)}} \times 100$$

Harvest

The limit of 45 days of cultivation was established, and the determination of the harvest point was performed visually, as described by Sturion (1994).

Statistical analysis

Bioestat 7.0 software was used to read the data. The experiment was completely randomized with twenty repetitions for each treatment. Means were compared between the same substrate types (pseudostem or stalk) and within substrates of the same banana cultivar. The ANOVA test was used to analyze variance, and the Tukey test was used to contrast means ($p < 0.05$).

RESULTS AND DISCUSSION

P. ostreatus NATB, is a native Amazonian mushroom that was grown on different substrates of two banana cultivars. It showed variation in the myceliation period and early formation depending on the substrate on which it was grown (Table 2). The substrates based on pseudostems led to a shorter myceliation period and consequent primordial formation. The difference on average between substrate types in relation to these parameters was 7 days.

Biological efficiency

The biological efficiency recorded for *P. ostreatus* in banana substrates ranged from 66.98% (PSPA) to 33.88% (ENTM). Analyzing Figure 1, the highest percentage of biological efficiency was achieved in pseudococcus substrate of silver-dwarf cultivar (66.98%), followed by the pseudostem of the thap-maeo cultivar (46%). The biological efficiency in the stems of these cultivars were similar: silver dwarf with 35.32%, followed by thap-maeo with 33.88%.

There was a statistical difference at the 95% probability level based on the Tukey test between the biological efficiencies of *P. ostreatus* cultivated in the pseudostems of the two cultivars tested and the difference in biological efficiency between the substrates of the silver-dwarf cultivar. The difference was not significant between the

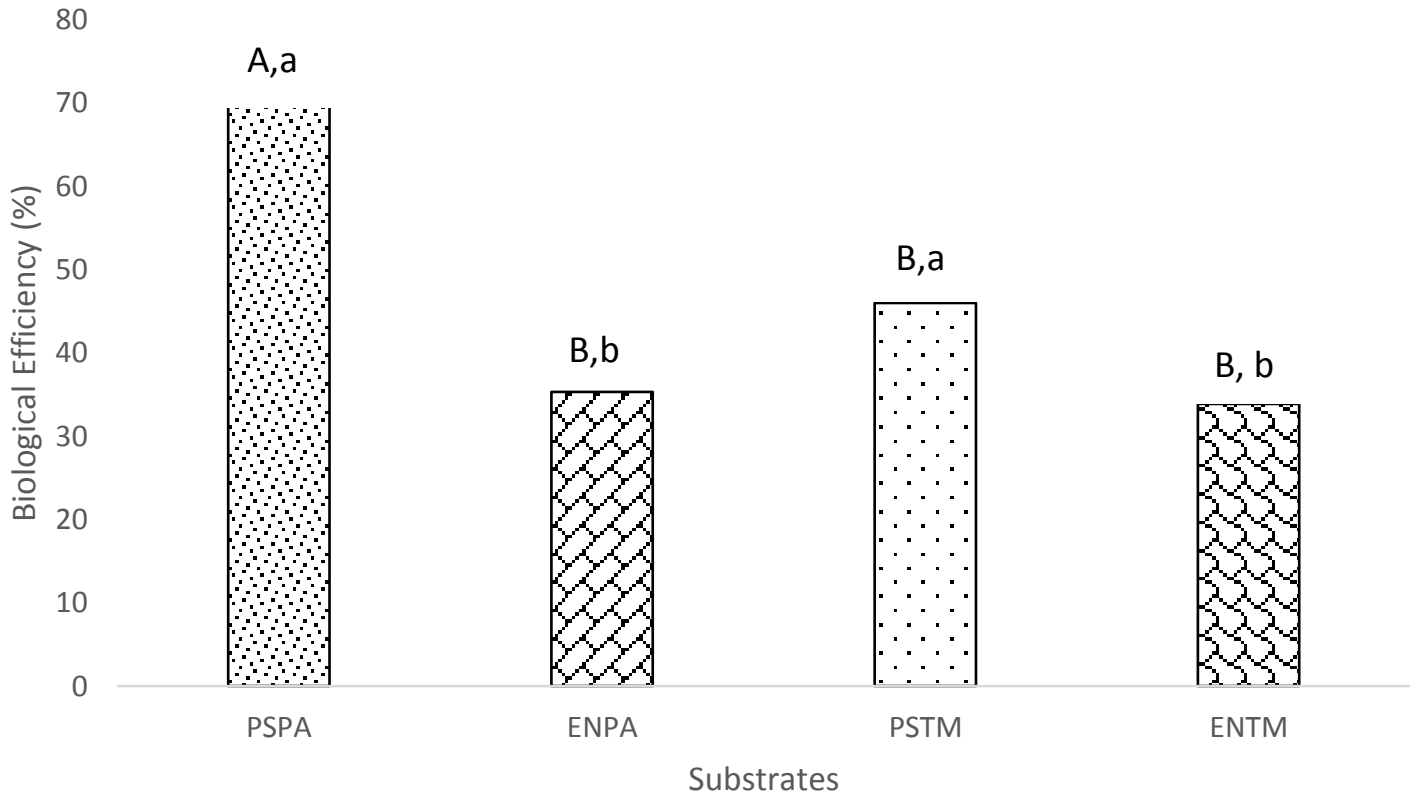

Figure 1. Average biological efficiency of the different substrates used in the cultivation of *Pleurotus ostreatus* NATB. PSPA: Dwarf silver pseudostem substrate; PSTM: Thap-maeo pseudostem substrate; ENPA: Thap-maeo stalk substrate; ENTM: Thap-maeo stalk substrate. Capital letters compare averages within the same type of banana substrate; Lower case letters compare averages within substrates of the same cultivar. Average of three repetitions. Means followed by equal letters do not differ from each other (Tukey, 5%).

pseudostem and thap-maeo stems ($p > 0.05$).

It was noted that the substrate composition influenced the productive parameters. According to Pedra and Marino (2006), this can be explained by the availability of nutrients that can be assimilated by the fungus. The treatments with pseudostems had the highest means and a positive effect of the substrates as a function of the analyzed parameter, indicating a greater dry mass bioconversion in carpophores especially for dwarf silver pseudostem production.

In a study carried out by Carvalho et al. (2012) that tested different cultivars and banana parts for *P. ostreatus* strain 09/100 production, the highest yield was observed in another substrate, thap-maeo of pseudostem (61.5%), leaving the dwarf silver pseudostem substrate at only 28% productivity.

Bonatti et al. (2003) used banana leaves to achieve much lower percentages for *P. ostreatus* (6.34%). Furlan et al. (2008) obtained 5.3 and 4.1% EB by cultivating *P. ostreatus* in cotton waste from the textile industry and in banana straw, respectively (Figure 1).

There are several factors that influence mushroom BE. Although the genus *Pleurotus* is quite versatile due to its ability to adapt to different substrate types and temperatures, substrate composition is important. Cultivation conditions or even lineage are parameters that can result in different production percentages. This study confirms the influence of the banana cultivar and its different substrates on *P. ostreatus* biological efficiency.

Yield

According to Figure 2, the highest average yield (g/kg) occurred on dwarf silver pseudostem substrate (139.95%). It was found that the average yield varied with the type of banana cultivar substrate used, and it was higher in pseudostem substrates.

The Tukey test ($p < 0.05$) showed a statistically significant difference between the types of residues and among the types of residues of the same cultivar.

Studies by Sales-Campos et al. (2010) that tested agroindustrial and timber residues for *P. ostreatus* production achieved higher percentages compared to our study. They obtained higher averages in crushed peach palm strain substrate (451.80 g/kg), followed by sugarcane bagasse substrate (250.40 g/kg) and marupá sawdust (242.80 g/kg). Yield data from a study using

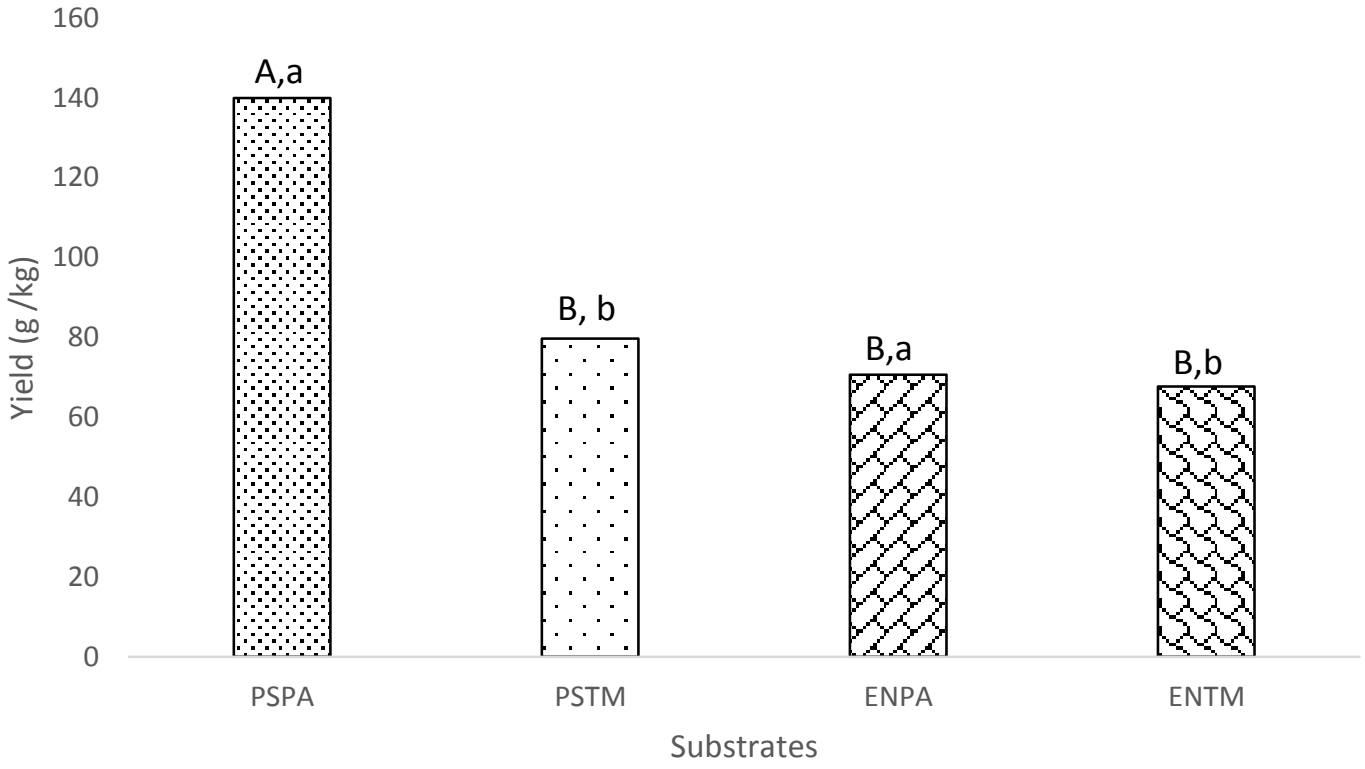

Figure 2. Average yield (g/kg) of the different substrates used in the cultivation of *P. ostreatus*. PSPA: Dwarf silver pseudostem substrate; PSTM: Thap-maeo pseudostem substrate; ENPA: Thap-maeo stalk substrate; ENTM: Thap-maeo stalk substrate. Capital letters compare averages within the same type of banana substrate; Lower case letters compare averages within substrates of the same cultivar. Average of three repetitions. Means followed by equal letters do not differ from each other (Tukey, 5%).

balsa wood sawdust was similar to those found for the dwarf silver pseudostem substrate (161.40 g/kg) (Figure 2). Rampinelli et al. (2010) tested banana straw in *Pleurotus djamor* cultivation, reaching yield percentages of 79.96% with 10% inoculum. Santos et al. (2000) also used banana straw to cultivate *P. sajor-caju*, reaching 93.03% yield in relation to dry substrate mass.

In this research, a positive correlation was observed between biological efficiency and average yield. Similar behavior was observed in studies by Sales-Campos (2008) using Amazonian timber and agroindustrial residues for the cultivation of *P. ostreatus.*

Loss of organic matter

Figure 3 shows the data on organic matter loss (PMO) of the different substrates used in *P. ostreatus* NATB cultivation. There was a statistical difference at the 95% probability level based on the Tukey test ($p < 0.05$) in organic matter loss and substrate type between the stems of the referred cultivars. When comparing the OML averages, there was a difference between the two types of residues of the same cultivar for both dwarf silver and Thap-maeo (Figure 3).

Duprat (2012) used peach palm leaf and rice bran supplementation as substrate for *P. ostreatus* DSM 1833 cultivation, achieving a percentage of 28.90% of OML, similar to the results found for dwarf silver pseudostem (28.92%). When the combination of substrate sheath and peach palm leaf (1:1) was tested with 20% inoculum, OML percentages of 36.8 were recorded for *P. ostreatus* DSM 1833; results that corroborate those observed for pseudostems prata-anã and thap-maeo of the present study.

Superior results were reported in a study by Carvalho et al. (2012) which tested four banana cultivars and different parts (pseudostem, leaf, pseudostem + leaf) in *P. ostreatus* 1467 cultivation. The authors obtained 61.5% of OML for the dwarf silver pseudostem substrate, followed by 57.3% for the thap-maeo pseudostem substrate. This differed from our results, registering 28.92% for dwarf silver pseudostem and 33.78% of OML for thap-maeo pseudostem substrate. This study shows that lower substrate OML correlates with higher biological efficiency BE.

Conclusion

P. ostreatus NATB successfully bioconverted plantain

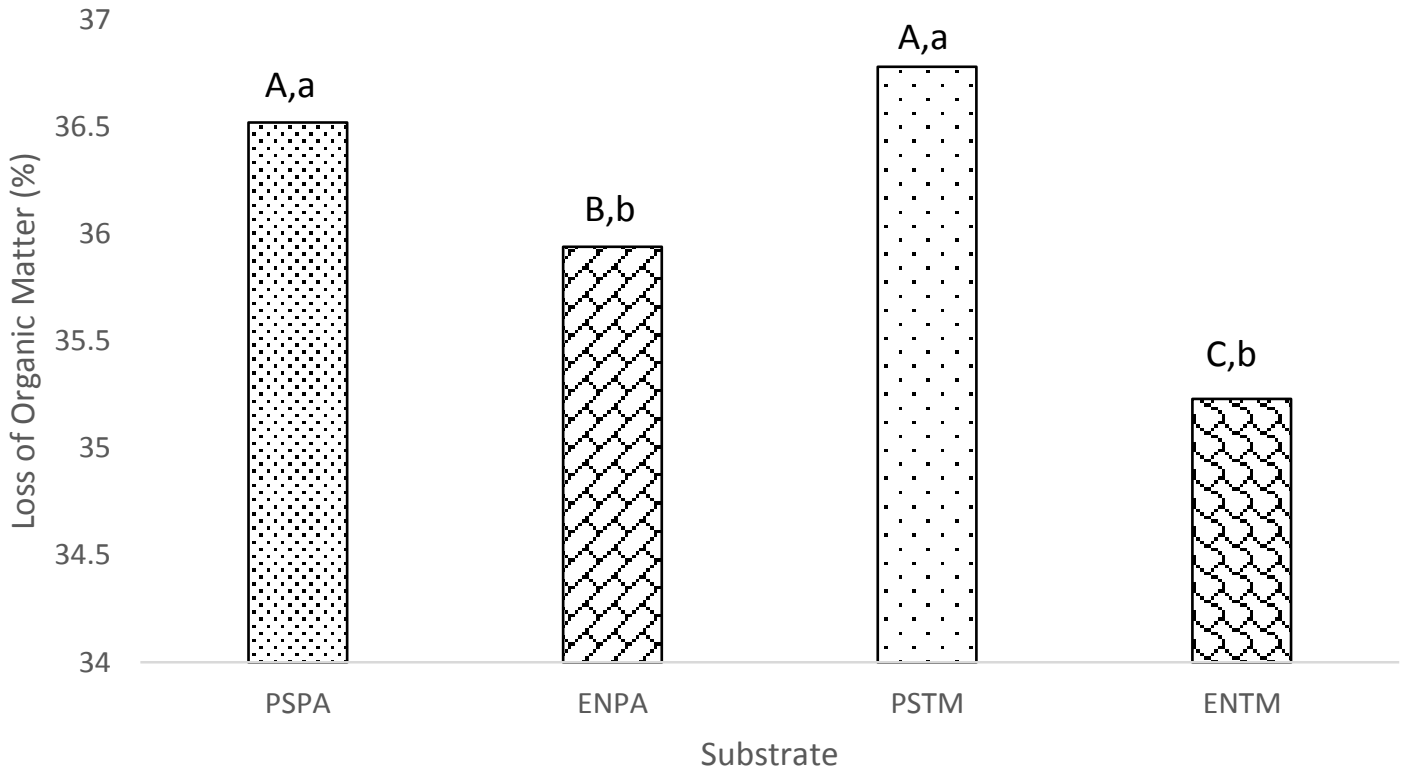

Figure 3. Loss of average organic matter from different substrates used in the cultivation of *P. ostreatus*. PSPA: Dwarf silver pseudostem substrate; PSTM: Thap-maeo pseudostem substrate; ENPA: Thap-maeo stalk substrate; ENTM: thap-maeo stalk substrate. Capital letters compare averages within the same type of banana substrate; Lower case letters compare averages within substrates of the same cultivar. Average of three repetitions. Means followed by equal letters do not differ from each other (Tukey, 5%).

residues, with emphasis on pseudostem production. The mushroom showed the best biological efficiency and yield on pseudostem substrates from the silver-dwarf cultivar. The substrates influenced the productive parameters of the evaluated mushroom.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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