DIVERSITY AND COLONIZATION OF ENDOPHYTIC MYCOFLORA AND STEM END ROT PATHOGEN, *Lasiodiplodia theobromae* [PAT.] GRIFF. AND MAUBL. IN MANGO cv. CARABAO

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ABSTRACT

The endophytic mycoflora harbors pathogenic and non-pathogenic fungi that affect the postharvest disease occurrence. This study explored the endophytic mycoflora in mango cv. Carabao that may affect the colonization of *Lasiodiplodia theobromae* [Pat.] Griff. and Maubl. Sequential sampling and isolation of pre-flowering, inflorescence, and fruit panicles of mango trees in dry and wet season production revealed the association of *L. theobromae* and eight other fungal genera with a total of 559 endophytic isolates. High endophytic mycoflora composition during the wet season by which *Penicillium* and *Aspergillus* exhibited the highest colonization frequency, possibly affected the endophytic colonization of *L. theobromae*. Fungal endophytes on 'Carabao' mango both for dry and wet seasons displayed low diversity (dry season-*H'*:1.93; wet season-*H'*:1.63). *L. theobromae* and endophytic mycoflora colonization showed positive correlation during the dry season and negative correlation during the wet season. Pathogenicity test revealed that endophytic *L. theobromae* isolates were pathogenic to mango fruits. This is the first report of endophytic colonization of *L. theobromae*, the dominant fungal pathogen causing stem end rot in mango cv. Carabao in the Philippines.

Key words: colonization rate and frequency, fruiting season, fungal diversity, pathogenicity

INTRODUCTION

Stem end rot (SER) is considered a major disease of mango similar to anthracnose. Preharvest fruit drop and postharvest rots are typical manifestations of SER disease. Infections remain latent at postharvest stage or until fruit senescence (Johnson et al. 1992b). The fungal rot is a postharvest disease with the typical rotting that initiates as brown to black discoloration at the stem end of the fruit. Rotting then progresses throughout the flesh of the fruit, thereby, affecting the quality, storage life, and transit of mango fruits leading to significant losses in mango production. Various fungal pathogens including *Dothiorella dominicana, D. mangiferae, Lasiodiplodia theobromae, Neofusicoccum* spp., *Phomopsis mangiferae, Cytosphaera mangiferae, Pestalotiopsis* sp., and *Alternaria alternata* are associated with SER disease (Alkan and Kumar 2018; Johnson et al. 1992a; Galsurker et al. 2018). In the Philippines, *L. theobromae* is known to be the main causal pathogen of SER. This fungal pathogen along with *Colletotrichum gloeosporioides* were consistently isolated in mango fruits sourced from major mango producing areas in the country (Lacambra 2005; Portales 2008). *L. theobromae* colonizes mango pedicels and inflorescences endophytically and eventually colonizes the fruit during ripening stage which results in SER infection (Johnson et al. 1992a; Diskin et al. 2017). Postharvest losses in mango do not result from infection at flowering or fruit set since fruitlets infected at this time are aborted (Johnson et al. 1992b).

Aside from the colonization of *L. theobromae*, several non-pathogenic microorganisms including fungal endophytes colonize mango stem endophytically. These endophytes reside inside healthy plant tissues without causing evident damage on the host (Stone et al. 2000). Promoting plant growth and providing protection against pathogens through different mechanisms are some of the benefits of these endophytes. A study on rice endophyte diversity reported that the frequency of colonization between sites, seasons, and varieties were found to differ significantly, which they attributed to the antagonistic properties of endophytes against fungal pathogens (Naik et al. 2009). Since the pathogenic *L. theobromae* and the fungal endophytes co-exist in the host tissues, the diversity of fungal endophytes might affect the endophytic colonization of *L. theobromae*. However, to the best of our knowledge,

there has been no studies conducted on the relationship between *L. theobromae* and the diversity of fungal endophytes in the different phenological stages of 'Carabao' mango. Microbial population prior to harvesting may affect the establishment of pathogens impacting on fruit quality (Bill et al. 2021). Thus, this study aimed to determine the correlation of diversity of fungal endophytes isolated from different growth stages to *L. theobromae* endophytic colonization in mango cv. Carabao in two fruiting seasons. Likewise, a pathogenicity test was carried out to determine if the endophytic *L. theobromae* isolates was able to cause SER disease in mango fruits.

MATERIALS AND METHODS

Sample collection. Experiments were conducted from 2018 to 2019 in a mango orchard in Los Baños, Laguna. Dry season coincided within the months of March to June while wet season occurred from October to January. Samples were collected in mango trees (10-20 years) at regular intervals in a mango orchard with moderate to high SER incidence. These mango trees were not sprayed with fungicides throughout the mango production. Two mango trees with uniform flowering were randomly chosen for the sequential sampling. Five healthy plant samples per tree were randomly collected at every growth stage (1: pre-flowering; 2: flowering; 3: fruit set; 4: young fruit (mungbean size); 5: fruit fill I (quail egg size); 6: fruit fill II (chicken egg size); and 7: late mature). These plant samples or panicles were divided into three parts particularly the top, middle, and bottom and for each part, five tissue segments (5mm long) were obtained for isolation. Tissue segments were processed employing the procedure of Johnson et al. (1992a) as adapted from Petrini (1986).

Isolation and identification. The tissue segments were triple sterilized (immersion in 95% ethanol for 60s, 2.5% sodium hypochlorite for 3 min and 95% ethanol for 30s) and inoculated in potato dextrose agar (PDA) amended with streptomycin sulfate (40ug/ml). The frequency of isolation of *L. theobromae* on tissue samples was noted. The endophytic fungi that were isolated in sequential sampling were characterized morphologically. The size, shape, and cultural growth of these fungal isolates were described. Each isolate was identified based on published literatures.

Colonization rate and frequency. Their occurrences in the sequential sampling were noted and correlated with *L. theobromae*. To determine the frequency of specific endophyte, present across all growth stages of mango, colonization frequency (CF) was calculated by the number of endophyte detections divided by the total number of segments for all growth stages multiplied by 100%. Fungal growth from the mango tissue planted onto the agar plate was considered an endophyte detection. To calculate the rate of colonization of all endophytes in a given growth stage of mango, colonization rate (CR) was determined as the number of detections of all endophytes divided by the total number of segments in that growth stage multiplied by 100%.

Diversity analysis. To determine the diversity of endophyte species, Shannon-Wiener diversity index was utilized using the formula:

$$H' = -\sum[(pi) * \log(pi)].$$

where pi was the proportion of endophyte of i-th species in the mango sample collections and was computed as pi = n/N. The n in the pi formula was the endophytes of a given species and N was the total number of endophytes in the mango sample collection. To compute for the Evenness, the formula used was:

$$E = H / \ln(k);$$

where H was the diversity value and k was the number of endophyte species.

Correlation analysis. To determine the effect of *L. theobromae* colonization to the fungal endophytes, Pearson correlation was computed for both wet and dry seasons using the fungal detection per growth stages. The correlation of *L. theobromae* and *Colletotrichum* sp., which incites anthracnose, was also calculated to determine if there is a positive or a negative correlation between the two pathogenic endophytes. The correlation of endophyte colonization and weather factors were also analyzed. Weather factors were correlated to endophyte incidence using the weather data from the Department of Science and Technology - Philippine Atmospheric, Geophysical and Astronomical Services Administration (DOST-PAGASA). The average temperature and rainfall (dry season: March to June 2018; wet season: October 2018 to January 2019) for each growth stage duration were used for the computation.

Pathogenicity of *L. theobromae* isolates. The *L. theobromae* isolates were subcultured in PDA for pathogenicity test. Asymptomatic mature 'Carabao' mango fruits were surface sterilized in running water and 70% ethyl alcohol. Fruits were pin pricked before inoculation. After airdrying, fungal growth was inoculated in mature mango fruits at nearly ripe stage (more yellow than green color in the peel). Inoculated fruits were incubated in moist condition by placing the fruits in blotter set-up. Lesion length was measured. Three fruits were inoculated per isolate. Re-isolation of inoculated fungal isolates was done. Colony and morphological characteristics of the re-isolated fungus were compared with the original culture. Means were compared through analysis of variance using least significant difference (LSD) (p-value < 0.05).

RESULTS AND DISCUSSION

Diversity and colonization analyses. Isolation from healthy plant tissues obtained a total of 559 endophytic fungal isolates in two mango fruiting seasons (dry season: 178; wet season: 381) belonging to 9 fungal genera (Table 1). These fungal endophytes belong to 9 fungal genera which include *Aspergillus, Colletotrichum, Curvularia, Epicoccum, Fusarium, Lasiodiplodia, Penicillium, Pestalotia,* and *Phomopsis*, which were all present in the dry and wet seasons except for *Curvularia, Epicoccum,* and *Phomopsis.* Among these fungal endophytes isolated, 4 genera are reported to cause SER namely *Lasiodiplodia, Phomopsis, Pestalotiopsis*, and *Dothiorella. L. theobromae* is considered the main causal organism of SER in the Philippines (Lacambra 2005; Portales 2008; Johnson et al. 1993). In some mango producing regions particularly in Asia and Australia, the main causal fungal pathogens were *Lasiodiplodia* and *Dothiorella* (Johnson et al. 1993). In Israel, the main pathogens causing SER are *Alternaria* and *Lasiodiplodia* (Diskin et al. 2017). This study presented only the mycoflora, however, it should be noted that various species of microorganisms including yeast and bacteria which may not be pathogenic are also present in the plant tissues (Galsurker et al. 2018). In addition, recovered major pathogenic fungal groups in mango including *Penicillium, Alternaria, Botryosphaeria*, and *Fusarium* as well as beneficial bacteria were also previously detected in several fruits such as avocado and grapes (Bill et al. 2021).

Considering the CF, the wet season showed higher frequency value (36.29%) than the dry season (16.95%) (Table 1 and Fig. 1). In terms of individual endophytes isolated, *Penicillium* has the highest CF value (14.86%) followed by *Aspergillus* (14.19%) across two fruiting seasons. The most frequently isolated endophyte in the dry season was *Penicillium* with CF of 5.52% while the most prevalent fungal endophyte in the wet season was *Aspergillus* exhibiting 12.67% colonization. It is interesting to note that *Penicillium* and *Aspergillus* had high CF during the wet season. Ubiquitous nature of these endophytes accounted to this result. Isolation from multiple and various plant parts revealed various endophytes especially *Penicillium* (Nicoletti et al. 2014). *Curvularia, Epicoccum* and *Pestalotia* have the lowest CF (0.10%). Seasonal change might affect colonization rate thus, resulting to difference in CF between the two fruiting seasons (Lodge and Cantrell 1995).

In terms of fruiting season, the wet season showed higher CF of *Lasiodiplodia* (3.24%). than the dry season (2.38%) (Table 1). This finding contrasts with the common observation that the dry season normally has a higher incidence of *Lasiodiplodia* or SER incidence than the wet season. It has been reported that SER infection may be higher than anthracnose disease in drier areas (Department of Agriculture and Fisheries-Queensland Government 2021; CABI 2021). The result in this study, however, was based on endophytic colonization and not on disease incidence on mango fruits. Likewise, results also suggested a higher detection of *Colletotrichum* than *Lasiodiplodia* during the wet season. This result is consistent with the common observation that anthracnose infection is expected to be higher when mango production coincides with the wet season, although this study did not assess disease infection during the postharvest stage.

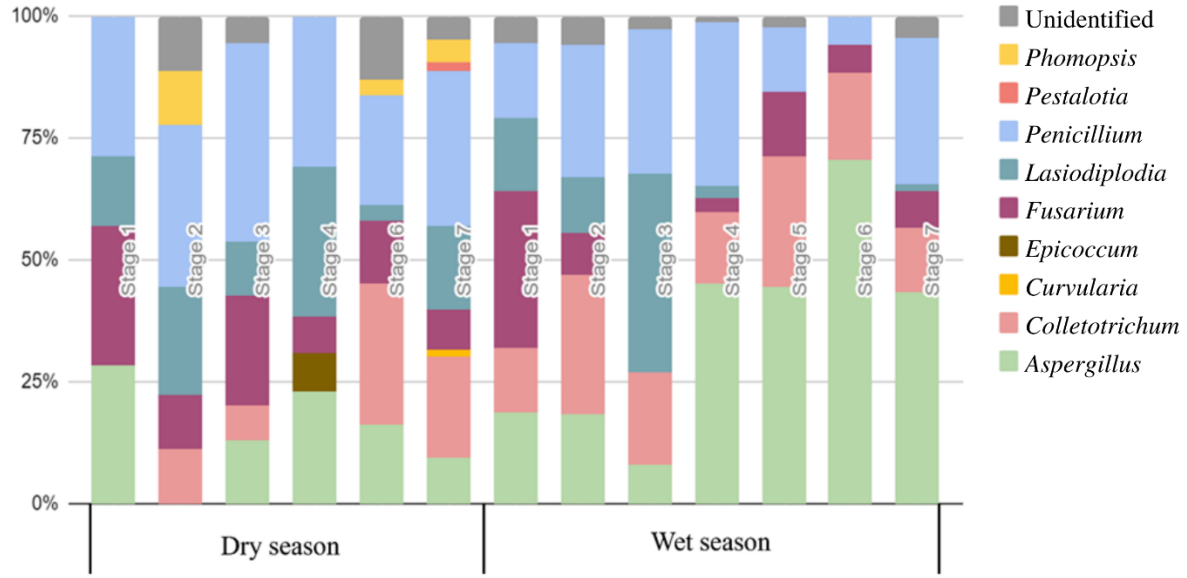

Fig. 1. Percentage of fungal endophytes detected during the dry and wet mango fruiting season 2018.

Table 1. Colonization frequency (CF) of endophytic fungi detected in mango cv. Carabao stem tissue from pre-flowering to late mature stages during the dry and wet seasons.

Endophytes	Dry Season									Wet Season								
	Growth stages*							Total detections	CF (%)	Growth stages*							Total detections	CF (%)
	1	**2**	**3**	**4**	**5**	**6**	**7**			**1**	**2**	**3**	**4**	**5**	**6**	**7**		
Aspergillus	2	0	7	3	0	5	6	23	2.19	10	13	3	34	20	24	29	133	**12.67*** **
Colletotrichum	0	1	4	0	0	9	13	27	2.57	7	20	7	11	12	6	9	72	6.86
Curvularia	0	0	0	0	0	0	1	1	0.10**	-	-	-	-	-	-	-	0	0.00
Epicoccum	0	0	0	1	0	0	0	1	0.10**	-	-	-	-	-	-	-	0	0.00
Fusarium	2	1	12	1	0	4	5	25	2.38	17	6	0	2	6	2	5	38	3.62
Lasiodiplodia	1	2	6	4	0	1	11	25	2.38	8	8	15	2	0	0	1	34	3.24
Penicillium	2	3	22	4	0	7	20	58	**5.52*** **	8	19	11	25	6	2	20	91	8.67
Pestalotia	0	0	0	0	0	0	1	1	0.10**	-	-	-	-	-	-	-	0	0.00
Phomopsis	0	1	0	0	0	1	3	5	0.48	-	-	-	-	-	-	-	0	0.00
Unidentified	0	1	3	0	1	4	3	12	1.14	3	4	1	1	1	0	3	13	1.24**
Total	**7**	**9**	**54**	**13**	**1**	**31**	**63**	**178**		**53**	**70**	**37**	**75**	**45**	**34**	**67**	**381**	

*Sequential sampling in different growth stages of mango cv. Carabao such as 1: pre-flowering; 2: flowering; 3: fruit set; 4: young fruit (mungbean size); 5: fruit fill I (quail egg size), 6: fruit fill II (chicken egg size), and 7: late mature.

**lowest CF

***highest CF

In terms of colonization rate (CR), stage 7 or the late mature stage of the mango fruit in the dry season and stage 4 or young fruit stage (mungbean size) in the wet season showed the highest CR value, 42% and 50%, respectively (Table 2). Stage 5 (fruit fill I - quail egg size) in the dry season (1%) and stage 6 (fruit fill II - chicken egg size) in the wet season (23%) exhibited the lowest CR value. These results imply that growth stages might not influence the colonization of fungal endophytes. Other factors including seasonal changes, weather factors, biochemical and physiological changes in fruiting stages, and mycoflora compositions possibly affect the endophyte colonization in mango.

Table 2. Colonization rate (CR) of endophytes isolated from various growth stages and fruiting seasons.

Growth Stages	Days After Flower Induction (DAFI)	Dry Season		Wet Season	
		Total detections*	CR (%)	Total detections*	CR (%)
Pre- flowering (1)	0	7	5.0 a**	53	35.0 a
Flowering (2)	27-28	5	6.0 a	70	47.0 a
Fruit set (3)	30-35	9	36.0 a	37	25.0 a
Young fruit (4)	40-45	54	9.0 a	75	50.0 a***
Fruit Fill I (5)	50-55	1	1.0 a	45	30.0 a
Fruit Fill II (6)	70-80	31	21.0 a	34	23.0 a**
Late Mature (7)	110-120	63	42.00 a***	67	45.0 a
Total	-	178	17.0	381	36.0

*Fungal isolates obtained from mango tissues planted on the agar media plates.
**Lowest CR
***highest CR

However, the differences in the CR for both seasons and for the seven (7) growth stages were not significant (Table 3). Endophytic outgrowth of SER fungi was reported in the inflorescence and mature stem tissues of mango (Johnson et al. 1993). Mango inflorescence had the richest fungal and bacterial communities at full bloom stage (Bill et al. 2021). Furthermore, a decline in fungal richness and diversity was lowest at the small size fruit stage similar to our observations. This finding can be attributed to the low sugar content in the pulp of immature fruit that gradually increases during fruit development (Quintana et al. 1984). Various physiological and biochemical changes during fruit development, such as activation of ethylene synthesis and other phytohormones, pH change, and decline of antifungal compounds might affect the mycoflora (Alkan and Fortes 2015). Likewise, this study presented an increase in fungal diversity at a late mature stage which is consistent with the findings of Bill et al. (2021).

The dry season (*H'*: 1.93) had a higher Shannon-Wiener diversity index (*H'*) value than the wet season (*H'*: 1.63) (Table 3). These diversity values were relatively low as species diversity commonly ranges from 1.5 to 3.5. Contrary to the species diversity value, wet season (0.84) has a higher Evenness (*E*) value than the dry season (0.91). These values strengthen the low diversity calculated for both fruiting seasons since evenness is inversely proportional to diversity. This contradicts to the previous findings that high fungal diversity is observed during wet or rainy seasons due to favorable conditions such as high humidity and low temperature (Mishra et al. 2012).

Table 3. Diversity of endophytes in the two fruiting seasons of mango cv. Carabao using Shannon-Wiener diversity index.

Indices	Dry season	Wet season
Shannon-Wiener diversity (*H'*)	1.93	1.63
Evenness (*E*)	0.84	0.91

Table 4. Correlation of *Lasiodiplodia theobromae* with fungal endophytes, and *Colletotrichum.*

	Lasiodiplodia	
	Dry season	**Wet season**
Endophytes	0.808	-0.461
Colletotrichum	0.675	0.095

Correlation analysis. The correlation between fungal endophytes and *L. theobromae* endophytic colonization showed contrasting results for both fruiting seasons. In the dry season, detection of *L. theobromae* showed positive correlation (0.808) to fungal endophytes colonization (Table 4). For instance, stage 7 (late mature stage) which showed the highest CR (42%) revealed that 11 isolates were *L. theobromae* and 52 isolates were endophytes. On the other hand, stage 1 (pre-flowering stage) having the lowest colonization rate (5%) obtained only one isolate of *L. theobromae*. All stages showed the same pattern in the dry season. However, the wet season showed a negative correlation (-0.461) between fungal endophytes and *L. theobromae* colonization. For instance, stages 4 (young fruit - mungbean size) to 7 (later mature fruit) showed relatively lower incidence of *L. theobromae* from 0 to 2 isolates, however, stages 4 (young fruit, mungbean size) and 7 (late mature fruit) had a CR of 50% (highest CR value) and 45%, respectively. Fruitlets of mango infected at flowering or fruit set are aborted (Johnson et al. 1992b). It is interesting to note that wet season displayed higher detection of endophytes than dry season. The negative correlation between *L. theobromae* and the fungal endophytes during wet season, and the high detection of fungal endophytes during this season probably is due to competition. Presence of dominating fungal endophytes including *Aspergillus* (CF: 12.67) and *Penicillium* (CF: 8.67) may have an adverse impact on *L. theobromae* endophytic colonization. Colonization by other endophytes and *C. gloeosporioides* in mango fruits from unsprayed trees could have delayed or prevented fruit colonization by SER fungi possibly due to competitive inhibition, antagonism, or induced host resistance (Johnson et al. 1992b).

Both fruiting seasons showed positive correlation (dry season:0.675; wet season:0.095) between the two pathogenic endophytes, *L. theobromae* and *Colletotrichum* (Table 4). This positive correlation indicates that both *L. theobromae* and *Colletotrichum* are co-colonizers and there is no competition between the two fungal endophytes. Both anthracnose and SER pathogens infect the stem end of mango fruit (Johnson et al. 1993).

Weather factors revealed consistent correlation of fungal endophytes to rainfall and temperature during the sample collection only for the dry season. Endophyte colonization and isolation rate showed positive correlation (0.21) to rainfall and temperature (0.22) during the dry season (Table 5). On the other hand, correlation of endophyte colonization to rainfall showed positive (0.32), however, its correlation (-0.32) to temperature revealed negative result during the wet season. These results signify that temperature and rainfall may influence the colonization and isolation rate of fungal endophytes especially during the wet season. However, considering other climatic conditions such as relative humidity, and moisture aside from temperature and rainfall might provide conclusive results as these conditions might affect also the endophytic colonization. Moreover, field experiments with various agro-climatic conditions is deemed necessary to further establish this assumption.

Table 5. Correlation of rainfall and temperature with endophyte colonization.

	Lasiodiplodia	
	Dry season	**Wet season**
Rainfall	0.21	0.32
Temperature	0.22	-0.32

Pathogenicity of *L. theobromae* isolates. Pathogenicity tests revealed that the *L. theobromae* isolates caused typical SER symptoms on mango fruits. Inoculation of these isolates caused dark brown to black rotting in ripe mango fruit (Fig. 2).

Fig. 2. Typical symptom of stem end rot during inoculation of endophytic *Lasiodiplodia theobromae* isolates in mango cv. Carabao fruits.

The mean lesion size induced by the isolates from various growth stages, ranged from 10.11mm to 32.08mm (Fig. 3). The dry and wet season isolates were confirmed to be pathogenic to mango fruit causing lesions ranging from 10.11 to 24.50mm and 12.50 to 32.08mm at 4 days after inoculation, respectively. No lesion size was indicated in the graph since no *L. theobromae* isolates were recovered during the fruit fill I in dry season as well as fruit fill I and II during wet season. *L. theobromae* isolates were successfully reisolated from these infected fruits. Re-isolated fungal cultures exhibited similar morphological and cultural characteristics with *L. theobromae*. This finding suggests that *L. theobromae* colonizes mango at various growth stages such that postharvest SER may occur if endophytic colonization reaches the stem end of the fruit. Results further confirmed reports that SER pathogens occur endophytically in mango. Fungicides and postharvest heat treatments may not easily eradicate the endophytic mycelium, however, the SER fungi occurring as pathogens could be limited by endophytes in the host plant (Johnson et al. 1993). The pathogenic capability of the isolates suggest that the fungus must be prevented or slowed down in reaching the stem end of the fruit before harvest to effectively control SER (Johnson et al. 1992b). This is the first report of endophytic colonization of *L. theobromae*, the dominant fungal pathogen causing stem end rot in mango cv. Carabao in the Philippines.

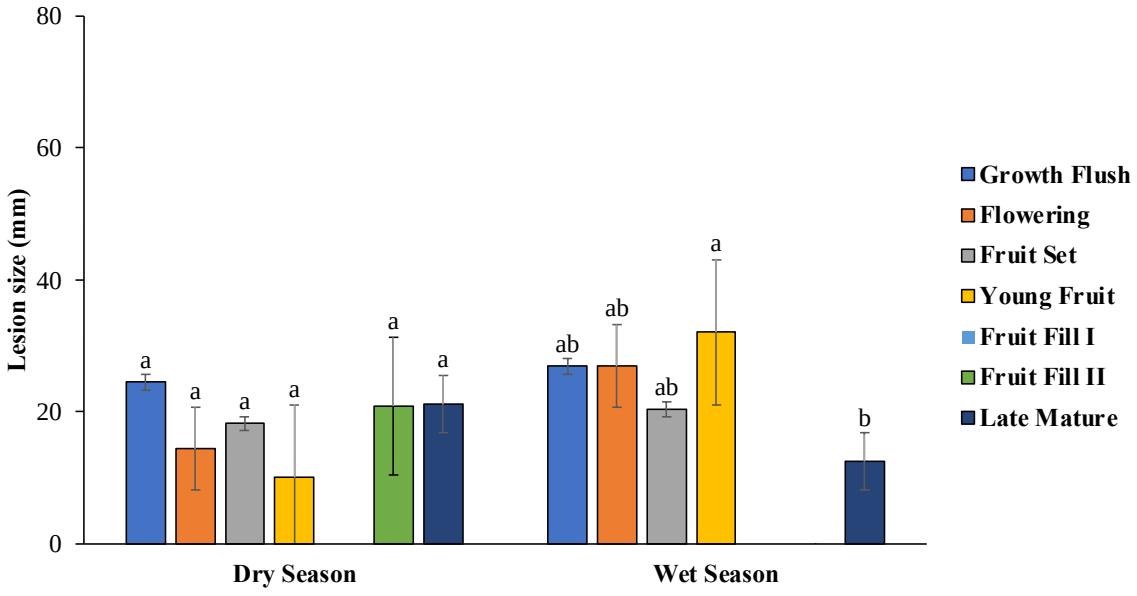

Fig. 3. Mean lesion size (mm) at 4 days after inoculation induced by endophytic *Lasiodiplodia theobromae* isolates from various growth stages in mango cv. Carabao fruits. Means with the same letter in each grouping are not significantly different in LSD (p-value = 0.05).

CONCLUSION AND RECOMMENDATION

Sequential sampling and isolation in pre-flowering, flower, and fruit panicles of mango trees in two fruiting seasons coinciding dry and wet season production obtained a total of 559 endophytic fungi belonging to 9 fungal genera. *Penicillium* (5.52%) and *Aspergillus* (12.67%) had the highest CF for the dry and wet seasons, respectively. Late mature fruit (dry season) and young fruit (wet season) displayed highest CR of 42% and 50%, respectively. Fruit fill I (dry season) and fruit fill II (wet season) exhibited the lowest CR value. Based on diversity index, both fruiting seasons obtained low diversity (dry season-*H'*:1.93; wet season-*H'*:1.63) of fungal endophytes.

Based on the data gathered in this study, wet season only affects the endophytic colonization of *L. theobromae* resulting to high endophytic mycoflora composition. Moreover, dry and wet seasons showed low diversity of fungal endophytes on 'Carabao' mango. Lastly, endophytic *L. theobromae* are pathogenic and can be a source of infection for SER in mango. The effect of application of systemic fungicide to the composition, diversity, and richness of fungal endophytes as compared to *L. theobromae* colonization is one of the future studies to explore. Lastly, the efficacy of fungal endophytes should also be studied to determine their potential as biological control agents against *L. theobromae* which can be utilized for disease management.

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