J.Natn.Sci.Foundation Sri Lanka 2014 42 (3): 229-240
DOI: http://dx.doi.org/10.4038/jnsfsr.v42i3.7396

RESEARCH ARTICLE

Effect of exogenous pH on development and growth of *Colletotrichum musae* and development of anthracnose in different banana cultivars in Sri Lanka

D.M. De Costa* and A.A.G. Chandima
Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Peradeniya.

Revised: 19 February 2013; Accepted: 09 May 2014

Abstract: In the present study, the relationship between the observed inter-cultivar variation of anthracnose development in banana by *Colletotrichum musae* and fruit peel pH was investigated. The results of a survey showed that anthracnose does not develop on Mondan (ABB), a cooking-type cultivar. In contrast, the highest susceptibility to anthracnose was reported by the dessert cultivars Anamalu and Embon (AAA). Variations of the pH of fruit peel exudates (FPE) of different banana cultivars were monitored during the ripening stages. The effects of FPE, collected from different banana cultivars at two maturity stages, on germ tube formation and elongation and appressoria development of *C. musae* were also determined. Furthermore, the effects of culture medium pH on the growth of *C. musae*, anthracnose development and the expression of a virulence-related gene, *pel*B were determined. With ripening the pH of FPE and fruit pulp decreased. The highest pH of FPE was shown by cultivars Seenikehel, Alukehel and Mondan with pH values greater than 6.0 at mature-unripe stage. At the ripe stage, pulp of all cultivars had pH values less than 4.5 with the lowest being 3.9 in Embul. When grown in culture media at a pH range from 4.0 – 8.0, the highest mycelial growth of *C. musae* was observed at 4.5, indicating that an exogenous pH of 4.5 can favour colonization by *C. musae*. The expression of *pel*B gene was detected by RT-PCR only in *C. musae* grown in a culture medium having a pH of 4.5. In addition, soluble solid content of the fruit peel was quantified as a possible factor responsible for the variation in anthracnose development among different banana cultivars, and its effects on germ tube formation, elongation and appressoria formation are discussed.

Keywords: Appressoria, fruit peel exudates, germ tube, *pel*B gene, RT-PCR.

INTRODUCTION

Anthracnose is considered as one of the most important and widely distributed diseases of banana at both pre- and postharvest stages. It is caused by the fungal pathogen, *Colletotrichum musae* (Berk. & Curt.) Arx. (Stover & Simmonds, 1987). Most of the commercial banana cultivars in Sri Lanka with higher consumer preference are susceptible to anthracnose, which causes considerable postharvest losses (Perera & Karunaratne, 1995; Perera *et al.*, 1999). The degree of susceptibility to anthracnose among the locally available banana cultivars has been observed to vary. However, the reasons for this variation are not clear. Although Perera *et al*. (1999) have reported variations in the physico-chemical properties of six locally available dessert banana varieties, a statistically significant correlation between those properties and the susceptibility to anthracnose has not been found. Nutritional compounds (e.g. sugars and amino acids) originating from the host plant can have positive or negative influences on the germination, growth and other stages of the infection process of plant pathogens (Carlile & Watkinson, 1994). Moreover, a wide range of organic and inorganic substances leached from living plant tissues or diffused into water droplets lying on the tissues promote fungal growth (Blakeman, 1975; Van Den Heuvel, 1987). Some postharvest fungal pathogens have the ability to enhance their virulence by locally modulating the host's ambient pH (Prusky *et al*., 2001). This mechanism ensures that virulence related genes coding for cell wall degrading and hydrolytic enzymes are expressed, and the relevant enzymes are secreted under the optimal pH conditions created by the pathogens by modifying the host pH. In *Colletotrichum* spp., avocado pathosystem, the alkalinization of host tissues (i.e. increasing of pH) during fruit ripening and the localized alkalinization induced by the pathogen's secretion of ammonia creates an appropriate pH for

* Corresponding author (devikadecosta@gmail.com)

expressing the *pel*B gene and secretion of its product, pectate lyase, a cell wall degrading enzyme (Prusky *et al.,* 2001; Yakoby *et al.,* 2001). A similar mechanism is involved with *Alternaria alternata* and this fungus also, induces the secretion of ammonia and enhances the pH needed for expression of the AaK1 gene coding for 1-4 β glucanase (Eshel *et al.,* 2002). In contrast, the secretion of oxalic acid during the infection of *Sclerotinia sclerotium* creates the acidic environment necessary for the activity of polygalacturanase (PG) produced by this fungus (Magro *et al.*, 1984). Moreover, the colonization of *Penicillium* spp. is enhanced by the low pH in host tissues (Prusky *et al*., 2002). Together these findings suggest that environmental pH is important as a global regulator for enhancing the virulence of several postharvest pathogens. Having this background, it was hypothesized that ambient host pH of peel tissues of banana has an influence on the growth, colonization, infection and anthracnose development of *C. musae*, and that the variation of host pH of banana peel tissue could be responsible for the observed variation in susceptibility to anthracnose among different banana cultivars.

Therefore, the objectives of the present study were to determine: (a) the variation of susceptibility to anthracnose; (b) the pH variation of fruit peel exudates (FPE) and pulp tissue during fruit ripening; (c) the effects of FPE collected from fully-mature and ripe banana on spore germination and appressoria formation of *C. musae*; (d) the effects of pH on growth of *C. musae in vitro* and anthracnose development *in vivo* and (e) the effects of pH on the expression of the virulence gene, *pel*B, in a range of dessert- and cooking-type banana cultivars in Sri Lanka. In addition, variation of the total soluble solid content (SSC) of fruit peel and pulp tissues of different banana cultivars were quantified over the fruit ripening period.

METHODS AND MATERIALS

Banana cultivars used in the experiment

Eight dessert banana cultivars and two cooking banana cultivars were used for the experiment. The genomic groups of the former were Embon (AAA), Anamalu (AAA), Embul (AAB), Seenikehel (ABB), Kolikuttu (AAB), Rathkehel (AAB), Puwalu (AAB) and Suwandel (AAB), and those of the latter were Alukehel (ABB) and Mondan (ABB). Mature, unripe fruits of each of the 10 cultivars (maturity index 1 according to standard banana ripening chart-Postharvest Technology Centre, University of California, Davis, USA) were collected from 14 different locations in 6 districts of Sri Lanka (Colombo, Galle, Matale, Kandy, Kegalle and Kurunegala) to determine the degree of susceptibility of the cultivars to anthracnose development. From each location 5 banana hands each having approximately 15 fruits per hand of each cultivar were collected from small-scale growers or from homegardens to ensure that no chemical applications had been carried out either at pre- or postharvest stages. For *in vivo* pathogenicity assay of *C. musae,* bananas were purchased from local growers who did not use any fungicides for disease control. From each cultivar 15 banana fingers (e.g. Alukehel, Embon, Kolikuttu, Embul and Seenikehel) were randomly selected from the purchased bunches and used for the inoculation of *C. musae*.

Colletotrichum musae cultures

C. musae was isolated from the dessert banana cultivar Kolikuttu showing typical anthracnose symptoms, which is highly susceptible to anthracnose. A pure culture of *C. musae* with cinnamon colour colony morphology (Photita *et al*., 2005) was obtained from a single cell culture and maintained on potato dextrose agar (PDA). The pure culture was stored using PDA discs of the fungal culture in sterile distilled water at 4 ^{o}C. Minimum sub-culturing was done during the storage period.

Determination of the degree of susceptibility to anthracnose development

Natural infections were allowed to develop by incubating the collected banana bunches (hands) from the 14 locations in an incubation chamber. The average maximum and minimum temperatures and relative humidity inside the chamber during the experimental period were 26 and 23 ^{o}C and 80 %, respectively. The incubation chamber used in the experiment was according to the description of Dillard (1989) with some modifications. The chamber was made up of glass (2×1.2×1 m) with a wooden frame and it could be closed completely to prevent moisture loss and temperature fluctuations. Fruits were placed on wire racks, which were kept on plastic trays lined with wet paper towels. The trays were staked to have adequate air circulation among them and two containers of hot water (having approximately 300 mL each) were placed inside the chamber to promote condensation and high relative humidity (near 100 %) as described by Dillard (1989). The natural infections of anthracnose developed on fruits were quantified as percentage disease incidence. The percentage anthracnose incidence for each cultivar at each location was calculated from a sample of 75 fruits as follows: (number of fruits showing typical anthracnose symptoms/number of fruits sampled)×100. These fruits were collected from the 14 locations as

described elsewhere in the text. Disease index was given for 50 fruits, which were randomly selected out of the 75 fruits, that were used to quantify disease incidence. The disease index was given on the basis of visually observed percentage disease severity as follows: 0 = no disease symptoms; 1 = less than 25 % of the fruit peel is covered with lesions; 2 = 25 − 50 % of the fruit peel is covered with lesions; 3 = 50 − 75 % of the fruit peel is covered with lesions and 4 = more than 75 % of the fruit peel is covered with lesions.

Collection of fruit peel exudates (FPE)

The fruits of banana cultivars Kolikuttu, Embon, Embul, Seenikehel, Alukehel and Mondan at two maturity stages (maturity indices 1 and 7 representing fully mature and ripe, respectively) were used to collect FPE. The above banana cultivars were selected to represent highly susceptible, moderately susceptible and least susceptible cultivars of banana based on visual observations of susceptibility to anthracnose (i.e. lesion development). According to initial visual observations, cultivars Kolikuttu and Embon were grouped as highly susceptible, Embul and Seenikehel as moderately susceptible and Alukehel and Mondan as least susceptible to anthracnose. Drops of sterilized distilled water (each having a volume of 250 µL), were placed on banana fruit peel at 3 − 5 different places per fruit. These fruits were incubated overnight in a humid chamber, which was a sealed plastic container lined with wet paper towels and having a container of water inside to avoid evaporation and maintain a near 100 % humidity (Dillard 1989; Wijesekara *et al*., 2011). About 10 mL of the FPE was collected from each cultivar × maturity stage combination (using approximately 7 − 8 fruits per combination) every day till the fruits became ripe (maturity index 7). On each day, collection of FPE was done from a fresh set of fruits incubated for the relevant number of days. The above collection procedure was repeated thrice per each cultivar × maturity stage combination. All peel exudates collected were separately filter-sterilized through a 0.2 µm filter and stored separately at 4 ^{o}C.

A volume of 5 mL of the un-filtered banana FPE collected daily from each cultivar × maturity stage combination and stored at 4 ^{o}C, was used to determine the pH of exudates.

Measuring the pH of peel exudates and fruit pulp

The pH values of the FPE and the fruit pulp of unripe to ripe stages in the six cultivars used to collect FPE were measured daily using a pH meter (TOA pH Meter HM-20SB, Japan). For measuring the pH of fruit pulp, fruit tissues of each cultivar × maturity stage combination were bored using a cork-borer and pH of the pulp was measured. Briefly, drops of sterile distilled water filled into the bored cavity of the fruit pulp were collected from a set of banana fruits (approximately 7 − 8) and the pH of the collected water was measured. Separate sets of fruits were used for daily measurements of the pulp pH. The above collection procedure was repeated thrice per each cultivar × maturity stage combination for pulp pH.

Quantification of the mycelial growth

To determine the effects of pH on the growth of *C. musae,* the fungus was grown initially in M_3S medium (primary culture) and then in a secondary medium having nine different pH levels ranging from 4 – 8 as described by Drori *et al.* (2003). M_3S medium contained the following reagents (per liter): 2.5 g $MgSO_4.7H_2O$, 2.7 g of KH_2PO_4, 1.5 g peptone Bios D (Biolife, Milan, Italy), 1.5 g Bacto yeast extract (Difco, Detroit, MI), 15 g sucrose, and 250 mg chloramphenicol. The fungus was grown in 60 mL of M_3S medium (Tu, 1985) in flasks of 250 mL at pH 5.0 and inoculated with 10^6 spores/flask by adding 1mL of *C. musae* spore suspension having a concentration of 10^6 spores/mL. Cultures were incubated at ambient temperature in a shaking incubator at 150 rpm for 4 days. Each culture was harvested by filtration through a sterile Buchner funnel fitted with filter paper (Whatman No. 1, Maidstone, England), and the hyphae remaining on the filter were washed twice, each time with 40 mL of sterile distilled water. The washed mycelia were re-suspended in 70 mL of fresh secondary medium (Drori *et al.,* 2003). The secondary medium contained the following reagents (per liter): 4 g of K_2HPO_4, 2 g of $MgSO_4.7H_2O$, 5 g of KNO_3, 0.3 g of $CaCl_2.2H_2O$, 10 mg of $FeCl_3$ and 10 g of glucose. To obtain initial pH values between 4.0 and 8.0, the culture medium was buffered with 0.05 M phthalate hydroxide buffer. The initial pH was determined after the medium was autoclaved but prior to inoculation. The pH was thereafter measured by separating 1 mL aliquots obtained at the required times after fungal inoculation. When needed, the pH was maintained at the desired value by adding an adequate amount of 0.05 M phthalate hydroxide buffer. All experiments were triplicated. The mycelial mat was harvested by filtering through a Buchner funnel fitted with filter paper (Whatman No.1, Maidstone, England). The weight of the mycelial mat was measured after air drying for 10 h.

Quantification of spore germination and appressoria formation of *C. musae* in peel exudates

A spore suspension of *C. musae* having a concentration of 10^5 spores/ mL was prepared under aseptic conditions

from a 12 − 13 day old culture of *C. musae.* The culture was vortexed in sterilized distilled water and filtered through several layers of muslin cloth to get a spore suspension. The spore concentration was adjusted to 10^5 spores/mL by using a haemocytometer. A 1 mL volume of the spore suspension was mixed with 1 mL of fruit peel exudates of different variety × maturity stage combination on cavity slides. Then the cavity slides were incubated in a humid chamber and spores with germ tubes and appressoria were counted in a unit area (0.5 mm^2) using 30 − 50 spores under a light microscope at a magnification of × 400 in time intervals of 2, 8 and 24 h after incubation at 24 °C.

Measurement of the germ tube elongation

Aliquots of *C. musae* spore suspension having 10^6 spores/mL were incubated in equal volumes of fruit peel exudates collected from 6 different banana cultivars (i.e. 4 dessert-types and 2 cooking-types) at two maturity stages as described in the section above. Elongation of germ tube length was measured at 2, 8 and 24 h after incubation at 28 °C at × 100 magnification using a calibrated occular micrometer. Randomly-selected 20 spores with germ tubes were measured to quantify the mean germ tube length.

Restoration of pH in fruit peel exudates

According to observations of the present study, the banana cultivar Kolikuttu at ripe stage showed the highest germ tube initiation and appressoria formation at the pH of the FPE collected. Hypothesising that the pH of the peel exudates of cultivar Kolikuttu is the most favourable for germ tube and appressoria formation, the exudate pH of each banana cultivar was adjusted to pH 5.73 (which is the pH of the exudates from Kolikuttu at ripe stage) using phthalate hydroxide buffer. The percentage number of germinated spores and spores with appresoria on pH adjusted-exudates were quantified.

In vivo* Pathogenicity assay of *C. musae

The *C. musae* cultures were grown in M_3S medium as described by Drori *et al.* (2003) at nine different pH values ranging from 4 – 8. The fungi were inoculated to fruits of mature banana cultivars Embon, Kolikuttu, Embul, Seenikehel and Alukehel. A 30 µL spore suspension with a concentration of 10^6 spores/mL was prepared and used to inoculate the healthy banana fruits at maturity index 1. Inoculation of *C. musase* was done by injecting the fruit peel with the spore suspension to a depth of 2 − 3 mm. Four points per fruit were inoculated. The inoculated fruits were arranged according to a completely randomized design (CRD). A control treatment was maintained in which *C. musae* was grown at neutral pH (pH = 7). All the inoculated fruits were incubated in the incubation chamber as described in the section on 'Determination of degree of susceptibility to anthracnose development' at ambient temperature, and near 100 % humidity for 5 − 10 days. Anthracnose development on fruits around each inoculated spot was quantified as the area of lesion in mm^2. The experiment was repeated twice.

Effects of pH on *pel*B gene expression

***Culturing of C. musae at different pH*:** A 40 mL volume of M_3S medium at pH 5.0 was inoculated with an aliquot of 500 µL of *C. musae* culture having a concentration of 10^6 spores/mL. Cultures were incubated at 24 °C with shaking at 150 rpm for 4 days. The mycelial mat was harvested by filtration using a Buchner funnel under aseptic conditions and the mycelium remaining on the filter paper was washed twice with 40 mL of sterile distilled water each time. The washed mycelial mat was re-suspended in 40 mL of fresh secondary medium (Drori *et al*., 2003). Nine such cultures were prepared having pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 in the secondary medium. pH of the secondary medium was adjusted to the above values using 0.05 M phthalate hydroxide buffer. These cultures were grown for four consecutive days and the pH values of the cultures were maintained by buffering with phthalate hydroxide buffer during the incubation period. At the end of the fourth day of incubation, mycelial mats were washed twice with sterilized distilled water, frozen with liquid nitrogen and stored separately at -80 °C for extraction of RNA. Total RNA of *C. musae* was extracted by the method described by Sokolovsky *et al.* (1990).

***Expression of pelB gene by RT-PCR*:** TaKaRa RNA PCR Kit (AMV) Ver. 3.0 was used to perform the reverse transcription of RNA to cDNA and subsequent amplification. Three hundred ng of total RNA, extracted by Sokolovsky *et al.* (1990) method from *C. musae* mycelia grown under different pH levels were used for each RT-PCR reaction. The primer for cDNA synthesis was a specific downstream primer. In the present study, 5'CACCAAGCCCGACTACAGCT3' and 5'AGCCTTACCTTGGAGGAGCC3' were used as the forward (upstream primer) and reverse primers (downstream primer), respectively (Kramer-Haimovich *et al*., 2006). RT-PCR reaction conditions were maintained for one cycle as given in the manufacturer's manual. A total of 40 µL reaction mixture was prepared as instructed by the manufacturer to perform the standard PCR. PCR was performed for 30 cycles, each cycle having a denaturing step at 94 °C for 30 s, annealing step

at 55 °C for 30 s and extension step at 72 °C for 30 s. After the amplification was completed, 5 µL of the PCR mixture was subjected to agarose gel electrophoresis to verify the amplified DNA fragments. A PCR fragment of approximately 1010 bp was expected.

Quantification of the total soluble solid content (SSC)

A homogenate was prepared from the fruit pulp and peel tissues of five different banana cultivars separately to represent different stages of the ripening period. Six different stages were identified according to the standard chart (Postharvest Technology Centre, University of California, Davis, USA) from maturity index 2 – 7. The homogenate was centrifuged at 3000 g for 5 min and a drop of the clear supernatant was used to measure the total soluble solid content in each sample using a hand held refractometer (Fisher Scientific, NY). The quantification was repeated two times and the measurements were taken in degrees Brix° (Perera *et al.,* 1999).

Statistical analyses

The effect of treatments (banana cultivars) on percentage disease incidence and disease index was determined by analysis of variance (ANOVA) and means were separated by least significant difference (LSD) (Kehinde, 2011). Significance or otherwise of the treatment effect on pH variation in FPE and in fruit pulp, growth of *C. musae*, germ tube elongation, area of anthracnose lesion development, soluble solid content in fruit peel and in fruit pulp was determined by ANOVA and mean separation was done by LSD.

RESULTS

Susceptibility status of different banana cultivars to anthracnose

The susceptibility status of different banana cultivars was determined based on two criteria, namely percentage disease incidence and disease index (Table 1). In terms of above criteria, the cooking cultivar Mondan had a 0 % disease incidence and a disease index of 0, indicating the total resistance to anthracnose. The highest disease index, which is a measure of the severity of anthracnose lesion development was recorded by the cultivars Embon (AAA) and Anamalu (AAA) while the rest of the cultivars (i.e. Suwandel, Kolikuttu, Rathkehel, Puwalu, Embul and Seenikehel) showed moderate disease indices.

Based on the disease incidence and disease index (in terms of percentage of peel tissue covered by anthracnose lesions), cooking-type cultivars showed a complete (e.g. Mondan) or very high resistance to the development of anthracnose (e.g. Alukehel). Among the dessert type cultivars, Embon and Anamalu showed a higher susceptibility while cultivars Embul, Seenikehel and Puwalu showed a moderate susceptibility to anthracnose (Table 1). Cultivars Kolikuttu and Suwandel (AAB) fell into two different susceptibility categories when their status was determined based on disease incidence and disease severity. As such, both cultivars belonged to the category showing the highest disease incidence, but moderate disease severity in comparison to cultivars Embon and Anamalu of the genomic group AAA.

Variation of pH of fruit peel exudates and fruit pulp

Table 1: Percentage disease incidence and disease index of anthracnose for different banana cultivars sampled from 14 locations in Sri Lanka

Cultivar /genomic group	% disease incidence	Disease index
Embon (AAA)	65.07 ^{a}	1.76 ^{a}
Anamalu (AAA)	66.67 ^{a}	2.00 ^{a}
Suwandel (AAB)	68.50 ^{a}	1.50 ^{ab}
Kolikuttu (AAB)	60.44 ^{a}	1.11 ^{ab}
Rathkehel (AAB)	42.00 ^{ab}	1.20 ^{ab}
Puwalu (AAB)	31.00 ^{ab}	1.00 ^{ab}
Embul (AAB)	21.36 ^{ab}	0.57 ^{ab}
Seenikehel (ABB)	17.14 ^{ab}	0.50 ^{ab}
Alukehel (ABB)	1.14 ^{b}	0.02 ^{b}
Mondan (ABB)	0.00 ^{b}	0.00 ^{b}

Note: In each column, means with the same letter are not significantly different at $p = 0.05$.

Changes of pH in fruit peel exudates and in fruit pulp of different banana cultivars over 11 days from mature unripe stage (maturity index 1) to ripe stage (maturity index 7) are shown in Figure 1. Cultivars Kolikuttu and Embul showed a higher ripening rate, hence the peel tissues were not available for measurement four days after the commencement of date of data collection.

The pH of FPE of all the tested cultivars except Seenikehel showed a decreasing trend (Figure 1 a) with ripening. In contrast, a significant increase of the pH was shown in the FPE of cultivars Seenikehel from unripe to ripe stages ($p < 0.0002$) (Figure 1a). The lowest FPE pH was shown by cultivar Embul at both unripe and ripe stages. The highest FPE pH (pH 6.76) at unripe stage was given by cultivar Mondan, but with ripening the pH decreased down to 6.1. The other cultivars had FPE

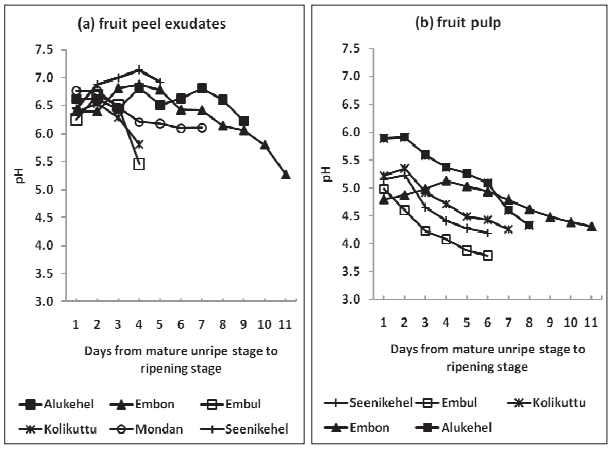

Figure 1: Variation of pH in fruit peel exudates (a) and fruit pulp (b) of different banana cultivars from mature unripe (maturity index 1) to ripe stage (maturity index 7).

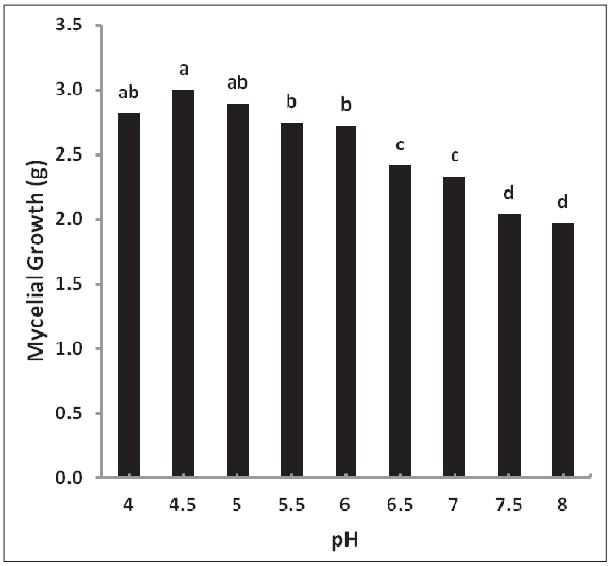

Figure 2: Air-dried weight of *C. musae* mycelium grown at different pH levels. Means with the same letter are not significantly different at p = 0.05.

pH values, which were between Mondan and Embul at unripe stage.

The variation of FPE pH from unripe to ripening stage of a given banana cultivar was different to the variation seen in fruit pulp pH (Figures 1a and 1b). In general, the pH values of FPE of all cultivars were higher than that of the pulp pH, both at unripe and ripe stages. The highest pulp pH at unripe stage was observed in cultivar Alukehel and the lowest in the cultivar Embon. At ripe stage the lowest pulp pH was shown by Embul and the highest by Embon.

All banana cultivars had significant differences between the pH values of FPE of unripe and ripe stages ($p < 0.0001$). In all varieties except Seenikehel, a lower FPE pH was observed at ripe stage than at unripe stage.

Growth of *C. musae* at different pH values

The pH of the culture medium strongly influenced the growth of *C. musae* ($p < 0.0001$) and the growth of *C. musae* was favoured by lower pH value of the culture medium. The highest mycelial growth was observed at pH 4.5 (Figure 2).

Formation of germ tube and appressoria from conidia of *C. musae*

Percentage germ tube formation was significantly influenced by the cultivar ($p < 0.0001$), maturity stage from which the FPE were collected ($p < 0.0001$), time of incubation in FPE ($p < 0.0001$), cultivar × maturity stage ($p < 0.0001$), cultivar × time of incubation ($p < 0.0001$), maturity stage × time of incubation ($p < 0.0002$) and cultivar × maturity stage × time of incubation ($p < 0.0001$) interactions. Similar influences were shown by the three factors (i.e. cultivar, maturity stage from which FPE was collected and the time of incubation) and the interactions of these factors ($p < 0.0001$) on percentage appressoria formed. *C. musae* did not form germ tubes in FPE collected from mature and ripe stages when observed two hours after incubation. In general, germ tube formation was lower in FPE collected from mature unripe fruits than in ripe fruits (Table 2). In addition, percentage germtube formation and appressoria formation showed an increasing trend with time of incubation in FPE collected from all banana cultivars, irrespective of the maturity stage at which FPE was collected. By the 24th hour after incubation, the highest percentage germination was shown when incubated in FPE collected from ripe stage of Kolikuttu. In contrast, the highest percentage germination of spores was shown by cultivar Embon, by the 24th hour after incubation when incubated in FPE collected at the unripe stage. The lowest percentage germ tube formation was shown in FPE of cultivars Alukehel, Seenikehel and Embul collected from ripe fruits.

In general, the appressoria formation was significantly lower in FPE collected from the mature unripe stage of all cultivars, in comparison to the ripe stage fruits of all cultivars (Table 2). The highest percentage appressoria formation was reported in FPE collected from mature unripe stage fruits of cultivar Embon, 24 hours after incubation. FPE collected from ripe fruits of cultivars Kolikuttu and Embon gave the highest percentage

Table 2: Percentage germ tube and appressoria formed by different banana cultivars in FPE of mature unripe and ripe stages at different periods of incubation

Percentage germ tube formation				
Cultivar	Mature unripe stage		Ripe stage	
	8 hrs	24 hrs	8 hrs	24 hrs
Alukehel	0.00 [b]	2.82 [b]	0.00 [d]	5.53 [d]
Mondan	0.00 [b]	5.01 [b]	0.00 [d]	20.86 [bc]
Embon	0.00 [b]	15.49 [a]	2.53 [bc]	30.86 [ab]
Kolikuttu	0.00 [b]	5.68 [b]	5.31 [a]	35.54 [a]
Embul	2.92 [a]	4.53 [b]	4.14 [ab]	10.54 [cd]
Seenikehel	0.00 [b]	2.03 [b]	1.37 [cd]	9.45 [cd]
Percentage appressoria formation				
Cultivar	Mature unripe stage		Ripe stage	
	8 hrs	24 hrs	8 hrs	24 hrs
Alukehel	0.00 [a]	0.00 [b]	0.00 [a]	2.70 [b]
Mondan	0.00 [a]	0.81 [ab]	0.00 [a]	8.26 [b]
Embon	0.00 [a]	3.17 [a]	0.00 [a]	22.77 [a]
Kolikuttu	0.00 [a]	1.15 [ab]	0.93 [a]	28.66 [a]
Embul	0.75 [a]	2.18 [ab]	0.00 [a]	5.27 [b]
Seenikehel	0.00 [a]	0.00 [b]	0.00 [a]	2.31 [b]

Note: Within each column, means with the same letter are not significantly different at p = 0.05.

appressoria formation at 24 hours of incubation (Table 2).

Eventhough germ tubes of spores were formed in FPE collected from mature unripe or ripe stages, it was observed that all germinated spores did not produce appressoria. The percentage of appressoria formed relative to the germinated spores was higher in FPE collected from fruits at ripe stage than in FPE collected from fruits at unripe mature stage (Table 3). The results showed that, FPE collected from unripe mature fruits of the cultivars Alukehel and Seenikehel promoted germ tube formation but not appressoria formation. In contrast, FPE collected from unripe mature fruits of cultivar Embul showed the highest percentage appressoria relative to the number of germinated spores. The highest percentage appressoria formation was reported in FPE from Kolikuttu collected at the ripe stage while the lowest was reported in FPE of the cultivar Seenikehel. The second highest value was reported by FPE of the cultivar Embon collected at the ripe stage and the second lowest was by the FPE of cultivar Embul.

Table 3: Percentage appressoria formed out of the germ tube formed conidia at 24 hours after incubation

Cultivar	Mature unripe stage	Ripe stage
Alukehel	0.00 [b]	55.56 [ab]
Mondan	16.67 [ab]	46.86 [b]
Embon	15.75 [ab]	76.25 [a]
Kolikuttu	33.33 [ab]	80.07 [a]
Embul	49.98 [a]	38.88 [c]
Seenikehel	0.00 [b]	27.79 [d]

Note: Within each column, means with the same letter are not significantly different at p = 0.05.

Germ tube length of *C. musae* conidia in FPE of different banana cultivars

The elongation of germ tube was influenced significantly by the banana cultivar ($p < 0.0001$) and the maturity stage ($p < 0.0001$), the period of incubation ($p < 0.0001$) and the interactions among the above three factors [(i.e. cultivar×maturity stage ($p < 0.0001$), cultivar × time of incubation ($p < 0.0001$), maturity stage × time of incubation ($p < 0.0001$) and cultivar × time of incubation × maturity stage ($p < 0.0001$)]. On average, for both maturity stages and all incubation time periods, germ tube elongation was highest in Kolikuttu followed by

Table 4: Germ tube length (μm) of *C. musae* conidia when incubated in FPE collected from different banana cultivars at two maturity stages and incubated at different time periods

Cultivar	Mature unripe stage			Ripe stage		
	2 hrs	8 hrs	24 hrs	2 hrs	8 hrs	24 hrs
Alukehel	0.00 [a]	6.65 [b]	18.40 [c]	0.35 [b]	12.00 [c]	47.35 [d]
Mondan	0.00 [a]	7.35 [b]	23.15 [bc]	0.35 [b]	14.00 [bc]	68.15 [cd]
Embon	1.35 [a]	16.50 [b]	51.40 [a]	3.10 [ab]	24.30 [ab]	136.00 [b]
Kolikuttu	1.05 [a]	50.60 [a]	61.20 [a]	10.70 [a]	33.50 [a]	204.00 [a]
Embul	0.00 [a]	14.25 [b]	32.10 [b]	0.70 [b]	21.70 [bc]	86.75 [c]
Seenikehel	0.00 [a]	28.55 [ab]	18.90 [c]	0.70 [b]	20.15 [bc]	49.15 [d]

Note: Within each column, means with the same letter are not significantly different at p = 0.05.

Embon. The lowest germ tube elongation was seen in Alukehel and Mondan. Cultivars Embul and Seenikehel showed moderate germ tube elongation (Table 4).

Germ tube and appressoria formation in FPE with adjusted pH

Adjusting the pH of FPE to that of FPE of the most susceptible cultivar (i.e. Kolikuttu) at ripe stage did not result in a significant increase of germ tube formation or appressoria formation ($p < 0.05$), suggesting that the higher percentages of germ tube formation and appressoria formation in cultivar Kolikuttu is not due to the pH of the FPE.

In vivo* pathogenicity assay of *C. musae

The area of anthracnose lesion development varied significantly among the banana cultivars tested ($p < 0.0001$), the pH at which the *C. musae* was grown ($p < 0.0001$) and the interaction effect of banana cultivar × pH of the growth medium of *C. musae* ($p < 0.0001$). In general, area of lesion development was highest in variety Kolikuttu while cultivars Seenikehel and Alukehel showed the lowest. When averaged across different varieties, the highest area of lesion development was shown by *C. musae* grown at pH 4.5 (Table 5). However, with respect to Kolikuttu, the highest area of lesion development was shown by *C. musae* grown at pH 5.0 (Table 5).

Table 5: Area of anthracnose lesion development (mm^2) on different banana cultivars when inoculated with *C. musae* cultured at different pH levels

pH	Alukehel	Embon	Kolikuttu	Embul	Seenikehel
4.0	1.47 ^{ab}	2.95 ^{abc}	3.52 ^{c}	3.13 ^{cd}	1.37 ^{ab}
4.5	1.98 ^{a}	3.49 ^{a}	4.15 ^{ab}	4.24 ^{a}	1.78 ^{a}
5.0	1.63 ^{a}	3.27 ^{ab}	4.25 ^{a}	4.03 ^{ab}	1.29 ^{bc}
5.5	1.01 ^{bc}	3.05 ^{abc}	3.94 ^{abc}	3.94 ^{ab}	0.96 ^{bcd}
6.0	1.01 ^{bc}	3.23 ^{ab}	3.71 ^{bc}	3.71 ^{b}	0.93 ^{bcd}
6.5	0.90 ^{c}	2.90 ^{abc}	2.88 ^{d}	3.19 ^{c}	0.85 ^{cd}
7.0	0.30 ^{d}	2.68 ^{bc}	2.77 ^{d}	2.79 ^{d}	0.72 ^{d}
7.5	0.15 ^{d}	2.46 ^{c}	2.72 ^{d}	1.74 ^{e}	0.66 ^{d}
8.0	0.06 ^{d}	1.71 ^{d}	2.46 ^{d}	1.29 ^{f}	0.16 ^{e}
no inoculation of *C. musae*	0 ^{d}	0.33 ^{e}	0.58 ^{e}	0.29 ^{g}	0 ^{e}

Note: Within each column, means with the same letter are not significantly different at $p = 0.05$.

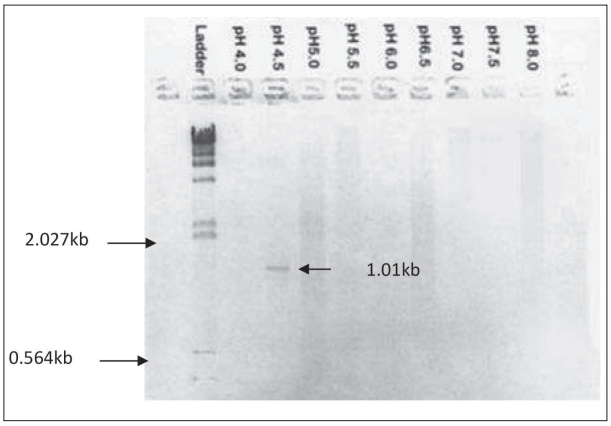

Figure 3: RT-PCR products produced by mRNA of *C. musae* grown at different pH levels. Size marker is λ/*Hind*III

Expression of *pel*B gene at different pH

Expression of *pel*B gene in *C. musae* mycelia grown at different pH levels was determined by RT-PCR. The PCR product with the expected size for *pelB* gene was given only by *C. musae* grown at pH 4.5. This indicates that the *pelB* gene expression is optimum in *C. musae* exposed to pH 4.5 (Figure 3).

Quantification of total soluble solid content

The total soluble solid content (SSC) in peel tissues of all banana cultivars showed a gradual decrease (Figure 4) with ripening. In contrast, the pulp tissues of different banana cultivars reported a gradual increase of SSC over the ripening period. The highest and lowest SSC were reported by the fruit peel tissues of cultivars

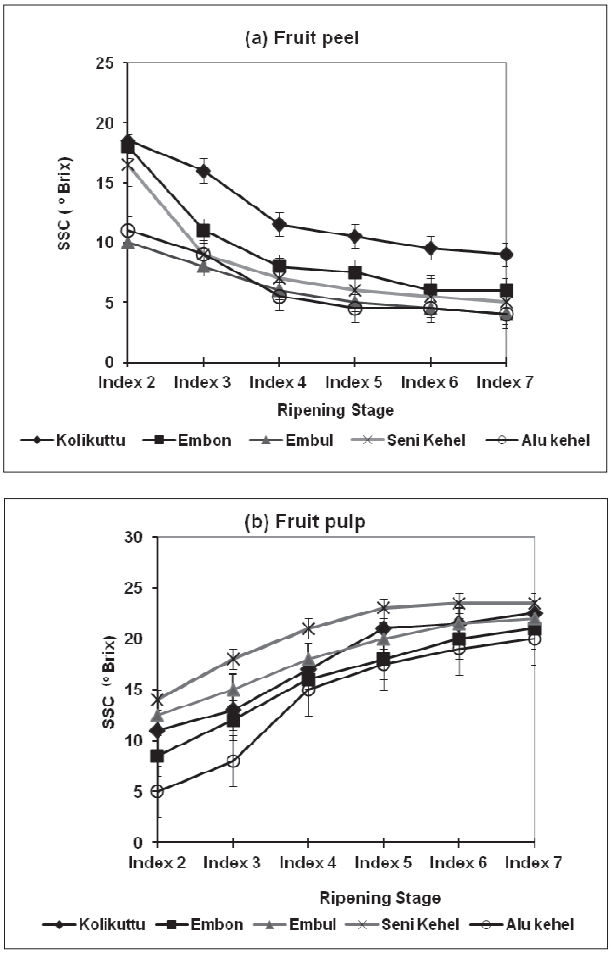

Figure 4: Soluble solid content (°Brix) on fruit peel (a) and fruit pulp (b) of different banana cultivars from mature unripe (maturity index 2) to ripe stage (maturity index 7)

Kolikuttu and Embul, respectively. The cooking type cultivar Alukehel also had a very low SSC in fruit peel. In contrast, SSC was highest in cultivar Seenikehel while cultivars Embul and Kolikuttu showed the next highest SSC in fruit pulp at the end of the ripening period. SSC was lowest in the pulp tissues of Alukehel, a cooking type cultivar.

DISCUSSION

Based on the two criteria that were used to determine the degree of susceptibility to anthracnose development, cooking and dessert type cultivars of banana used in the present study could be grouped into resistant, least-, moderate- and highly-susceptible cultivars. Accordingly, Mondan, a cooking type cultivar was resistant to anthracnose while Alukehel showed the least susceptibility to anthracnose. Dessert-type cultivars (i.e. Rathkehel, Puwalu, Embul and Seenikehel) were moderately susceptible in terms of disease incidence and severity. However, cultivars Kolikuttu and Suwandel had a higher anthracnose incidence but lesion development (severity) showed moderate susceptibility. This is a possibility as there could be cultivars with slower spreading rates of the lesions due to inherent host plant characteristics, despite getting infected and reporting a higher disease incidence. The results showed that the banana cultivars having ABB genomes were either resistant or least susceptible to anthracnose development whereas cultivars of AAA genomes were highly susceptible. The cultivars having AAB genomes showed a moderate susceptibility to the disease. This could be due to the fact that varieties with an AAB or ABB genome constitution are more tolerant and hardy to biotic and abiotic stress factors owing to the presence of the B genome (Robinson & Sauco, 2010).

The present study showed the variation of pH of FPE and fruit pulp during ripening of a range of banana cultivars grown in Sri Lanka, and highlighted their possible influence on anthracnose development. All the tested banana cultivars showed a range of pH values from 3.79 – 4.31 in pulp tissues at the ripe stage. This is in contrast to the increased pH or alkalinisation of the fruit tissues that occurs during natural ripening where pH of the pericarp of avocado increases from 5.2 – 6.1 (Kramer-Haimovich *et al.*, 2006). However, banana is an exception because the highest acid levels are attained when the fruit is fully ripe, with the pH declining from *ca.* 5.4 ± 0.4 at the pre-climacteric stage to *ca.* 4.5 ± 0.3 at the post-climacteric pulp (Palmer, 1971). The pH values of the ripe pulp tissues obtained in the present study for cultivars Embul, Embon, Kolikuttu, Seenikehel are on par with the observations of Perera *et al.* (1999).

The influence of fruit tissue pH as a regulatory cue for processes linked to the pathogenicity of postharvest pathogens and also for the expression of pathogenicity-related genes of those pathogens has been well documented on several pathosystems (Eshel *et al*., 2002; Drori *et al*., 2003; Prusky *et al*., 2004). Findings of the present study support the fact that acidic conditions in the host tissues favour mycelia growth of *C. musae*, especially at lower pH (4.0 – 5.0) and promote expression of the *pel*B gene at pH 4.5. The need of a low-pH environment for infection and pathogenicity related activities agree with the findings on *Penicillium digitatum*, a pathogen specific to citrus fruit in which spore germination and mycelia growth are stimulated in low pH environments (4.0 − 5.5) (Pelser *et al.,* 1977; French *et al.,* 1978).

However, the findings of the present study differ from the existing knowledge of pathogenicity of *Colletotrichum gloeosporioides* in avocado, *C. coccodes* in tomato and *C. acutatum* in apple where the expression of virulence factors and the *pelB* gene of the pathogen is encouraged by alkalinization of the fruit tissues due to the natural ripening process or by the influence of the pathogen (Yakoby *et al.,* 2000, 2001; Prusky *et al*., 2001; Drori *et al*., 2003).

Eventhough the change of banana pulp pH has been studied in detail, we believe that this is the first study to investigate the variation of FPE pH in different banana cultivars including dessert and cooking types during ripening. Such information revealed important findings on the impact of early developmental stages of the infection process of *C. musae* and possible reasons for the variations of anthracnose development between different banana cultivars.

Findings of the present study also indicated that the host tissue environment with a lower pH could not be the sole factor favouring anthracnose development of banana. This is because, in the variety Embul, which had a lower FPE pH value, anthracnose development was not as high as in the other varieties with lower FPE pH values. In contrast, findings of this study confirmed that the higher spore formation and appressoria formation in FPE collected from ripe fruits of the variety Kolikuttu was not only due to its pH. Hence, stimulatory effects of some other plant originated substances are possible, especially during early steps of the infection process (e.g. spore germination and appressoria formation). The present study clearly showed that the germination of spores, elongation of germ tube and formation of appressoria were highly dependent on the banana cultivar and the maturity stage at which FPE was collected. Similar observations have been reported by Wijesekara *et al.* (2011) on the stimulation of conidia germination and appressoria formation of *C. gloeosporioides* on fruit exudates of Uguressa [*Flacourta indica* (Burmm.F). Merr].

Such stimulatory effects on conidia germination and appressoria formation could be due to the variations in total soluble solid content (SSC) in fruit peel tissues of different banana cultivars. The positive influence of fructose, glucose and sucrose on conidia germination of *Botrytis cinerea* at concentrations above 100 mM has been reported by Nassr and Barakat (2013). Brix values for SSC obtained for the fruit pulp of different dessert type banana cultivars in the present study agree with the results of Perera *et al.* (1999). However, no information is available for a comparison of the Brix values of FPE, and it is believed that this is the first study focusing on SSC of FPE of different banana cultivars. The information on chemical properties of FPE would be more useful as fruit peel is the initial site of infection on which the conidia land, germinate and the infection process starts. Brix values of the fruit peel in all banana cultivars revealed a decrease during ripening while the fruit pulp showed an increasing trend. Based on this information, higher conidia germination and appressoria formation in cultivars Kolikuttu and Embon could be due to the presence of higher SSC in the peels of these cultivars throughout the ripening phase. Higher germ tube length in FPE of cultivar Kolikuttu could also have been similarly influenced by the nutrient and chemical composition of its FPE. In contrast, germ tube formation, appressoria formation and germ tube length in the FPE of cultivar Embul were lower eventhough the pH of its FPE was the lowest at the ripe stage. This could be due to the lowest Brix value of the fruit peel of cultivar Embul. As FPE of cultivar Embul does not favour germ tube formation, appressoria formation and germ tube elongation to the same extent as in cultivars Kolikuttu and Embon, lower anthracnose development could be expected.

Findings of the present study support the view that different chemical properties such as pH and SSC of the fruit peel are responsible for the variation in anthracnose development among different banana cultivars. The variation of anthracnose development among different banana cultivars could be a result of the differential influence of the above chemical properties of the FPE on germ tube formation (i.e. conidia germination), appressoria formation, germ tube elongation, colonization, and expression of the *pel*B gene during the infection process of *C. musae*. Based on the results of this study, it is possible to conclude that early steps of the infection process, which occur on the fruit peel such as germ tube formation (i.e. conidia germination), appressoria formation and germ tube elongation, are influenced by the SSC of the FPE. On the other hand, the subsequent steps of the infection process such as mycelia colonization and *pel*B gene expression are influenced by the pH of the fruit tissues.

CONCLUSION

Based on the above findings it can be concluded that

- Cooking type banana, Alukehel (ABB) and dessert-type banana, Seenikehel (ABB) and Embul (AAB) can be considered as less and moderately susceptible cultivars to anthracnose, respectively at the postharvest stage. Anthracnose did not develop at the postharvest stage on Mondan (ABB), a cooking type banana cultivar.

- Exogenous pH of 4.5 favoured the pathogenicity of *C. musae* in banana by promoting fungal growth, development of anthracnose lesions and expression of *pelB* gene.
- In addition to the pH of FPE, SSC of the fruit peel is a possible factor responsible for the variation in anthracnose development among different banana cultivars due to effects on germ tube formation, elongation and appressoria formation.

Acknowledgment

Authors wish to acknowledge the financial support by the International Science Foundation, Sweden (Grant no. E/3349-2) and the statistical advice of Prof. W.A.J.M. De Costa, Department of Crop Science, University of Peradeniya, Peradeniya.

REFERENCES

1. Blakeman J.P. (1975). Germination of *Botrytis cinerea* conidia *in vitro* in relation to nutrient conditions on leaf surfaces. *Transactions of the British Mycological Society* **65**: 239 − 247.
2. Carlile M.J. & Watkinson S.C. (1994). *The Fungi*. Academic Press, London, UK.
3. Dillard H.R. (1989). Effect of temperature, wetness duration, and inoculum density on infection and lesion development of *Colletotrichum coccodes* on tomato fruit. *Phytopathology* **79**: 1063 − 1066. DOI: http://dx.doi.org/10.1094/Phyto-79-1063
4. Drori N., Kramer-Haimovich H., Rollins J., Dinoor A., Okon Y., Pines O. & Prusky D. (2003). External pH and nitrogen source affect secretion of pectate lyase by *Colletotrichum gloeosporioides*. *Applied and Environmental Microbiology* **69**: 3258 − 3262. DOI: http://dx.doi.org/10.1128/AEM.69.6.3258-3262.2003
5. Eshel D., Miyara I., Ailinng T., Dinoor A. & Prusky D. (2002). pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruits. *Molecular Plant-Microbe Interactions* **15**: 774 − 779. DOI: http://dx.doi.org/10.1094/MPMI.2002.15.8.774
6. French R.C., Long R.K., Latterell F.M., Graham C.L., Smoot J.J. & Shaw P.E. (1978). Effect of nonanal, citral, and citrus oils on germination of conidia of *Penicillium digitatum* and *Penicillium italicum*. *Phytopathology* **68**: 877 − 882. DOI: http://dx.doi.org/10.1094/Phyto-68-877
7. Kehinde I.A. (2011). Response of melon cultivars to natural infection by diseases in South Western Nigeria. *International Journal of Biology* **3** (4): 47 − 55. DOI: http://dx.doi.org/10.5539/ijb.v3n4p47
8. Kramer-Haimovich H., Servi E., Katan T., Rollins J., Okon Y. & Prusky D. (2006). Effect of ammonia production by *Colletotrichum gloeosporioides* on *pelB* activation, pectate lyase secretion, and fruit pathogenicity. *Applied and Environmental Microbiology* **72**(2): 1034 − 1039. DOI: http://dx.doi.org/10.1128/AEM.72.2.1034-1039.2006
9. Magro P., Marciano P. & Di Lenna P. (1984). Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. *FEMS Microbiological Letters* **24**: 9 − 12.
10. Nassr S. & Barakat R. (2013). Effect of factors on conidium germination of *Botrytis cinerea in vitro*. *International Journal of Plant and Soil Science* **2**(1): 41 − 54.
11. Palmer J.K. (1971). The banana. *The Biochemistry of Fruits and their Products* (ed. A.C. Hulme), pp. 65 − 105. Academic Press, London, UK.
12. Pelser P., du T. & Eckert J.W. (1977). Constituents of orange juice that stimulate the germination of conidia of *Penicillium digitatum*. *Phytopathology* **67**: 747 − 754. DOI: http://dx.doi.org/10.1094/Phyto-67-747
13. Perera N. & Karunaratne A.M. (1995). A study of some peel characteristics of five local varieties of banana and non-pesticidal chemicals that promote resistance to postharvest diseases. *Proceedings of Sri Lanka Association for the Advancement of Science* **51**(1): 68 − 70.
14. Perera O.D.A.N., Basnayake B.M.K.M.K. & Karunaratne A.M. (1999). Physiological characteristics, popularity and susceptibility to anthracnose of some local banana cultivars. *Journal of the National Science Foundation of Sri Lanka* **27**(2): 119 − 130.
15. Photita W., Taylor P.W.J., Ford R., Hyde K.D. & Lumyoung S. (2005). Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Diversity* **18**: 117 − 133.
16. Prusky D., McEvoy J.L., Leverentz B. & Conway L.S. (2001). Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. *Molecular Plant-Microbe Interactions* **14**: 1105 − 1113. DOI: http://dx.doi.org/10.1094/MPMI.2001.14.9.1105
17. Prusky D., McEvoy J.L. & Conway W.S. (2002). Local pH modulation by pathogens as a mechanism to increase virulence. *6th European Conference on Fungal Genetics*, Pisa, Italy, p. 319.
18. Prusky D., McEvoy J.L., Saftner R., Conway W.S. & Jones R. (2004). Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. *Phytopathology* **94**: 44 − 51. DOI: http://dx.doi.org/10.1094/PHYTO.2004.94.1.44
19. Robinson J.C. & Sauco V.G. (2010). *Bananas and Plantains,* 2nd edition. CABI Publishing. Wallingford/Oxon, UK. DOI: http://dx.doi.org/10.1079/9781845936587.0000
20. Sokolovsky V., Kaldenhoff R., Ricci M. & Russo V.E.A. (1990). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*. *Fungal Genetic Newsletter* **37**: 41 − 43.
21. Stover R.H. & Simmonds N.W. (1987). *Bananas,* 3rd edition, pp. 468. Longman, London, UK.
22. Tu J.C. (1985). An improved mathur's medium for growth, sporulation and germination of spores of *Colletotrichum lindemuthianum*. *Microbiosis* **44**: 87 − 93.
23. Van Den Heuvel J. (1987). Substances in dead plant tissue that stimulate infection of French bean leaves by *Botrytis cinerea*. *Netherlands Journal of Plant Pathology* **93**: 135 − 146.

DOI: http://dx.doi.org/10.1007/BF02000564

24. Wijesekara W.M.A.U.K.M., Rajapakse R.G.A.S. & Dissanayake M.L.M.C. (2011). Effect of fruit and leaf exudates for the development of anthracnose and rust pathogens in uguressa, *The Journal of Agricultural Sciences* **6** (2): 77 − 83.
25. Yakoby N., Beno-Moualem D., Keen N.T., Dinoor A., Pines O. & Prusky D. (2001). *Colletotrichum gloeosporioides pel*B, is an important factor in avocado fruit infection. *Molecular Plant-Microbe Interactions* **14**: 988 − 995. DOI: http://dx.doi.org/10.1094/MPMI.2001.14.8.988
26. Yakoby N., Kobiler I., Dinoor A. & Prusky D. (2000). pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. *Applied and Environmental Microbiology* **66**: 1026 − 1030. DOI: http://dx.doi.org/10.1128/AEM.66.3.1026-1030.2000