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# RESPODIP TREATMENT WITH NANO EMULSION OF HEXANAL TO REDUCE THE ANTHRACNOSE DISEASE OF BANANA AND EXTEND ITS SHELF- LIFE

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## ABSTRACT

**TBanana is an important crop that meets the economy and food security of many countries. Anthracnose caused by *Colletotrichummusae*(Berk & M.A. Curtis) an important post-harvest disease in banana causing huge economic drain.To manage this disease, several chemical fungicides are used so far and indiscrimate use had resulted in health and environmental concern which warrants an alternate method of biological control. Hexanal, a natural plant volatile compound was shown to extend the shelf life fruits by inhibiting the Phospholipase enzyme D (PLD). In this study the antifungal activity of the hexanal was studied. Fresh bunches of GrandNaine banana fruits were harvestedat 80 per cent maturity and thentreated with two percent Nano emulsion of hexanal as Enhanced Freshness Formulation (EFF) in the form of dip treatment. Dip treatment with EFF for 5 minutes effectively controlled the germination of the conidia and prevented the germ tube formation of the pathogen. The dip treated fruits remained green up to 12 days after treatment, while the control fruits (without EFF dip treatment) rot on 7$^{th}$ day. In addition, we observed that the EFF dip treated fruits showed enhanced lignification, which prevented the invasion of the pathogen. Moreover, the defense enzymes like poly phenol oxidase, phenylammonialyase and catalase accumulation was higher up to 7$^{th}$ day and gradually decreased. Thus, the hexanal in the form of EFF dip treatment for 5 minutes reduced the post-harvest disease anthracnose and extended the shelf life of banana variety Grand Naine up to 12 days by increased accumulation of defense enzymes.**

**(Key words:Post harvest disease, Anthracnose, hexanal, EFF dip treatment, defense enzymes)**

## INTRODUCTION

Banana (*Musa* sp.) is the most important tropical fruit crop in the world, occupying eighth position among the food crop (Ploetz, 2015). Banana is rich in nutrients, minerals and vitamin C with the antioxidant level up to 15 % (Natalia Suseno *et al.,* 2014). It grows well in the tropical climate and its climacteric nature have some serious limitations that leads to the deterioration of the fruits by the infection of post-harvest pathogens. Post harvest decay provocative by the fungal pathogens is one of the major limiting factor for the economic values of banana fruits (Palou *et al.,* 2016) and post-harvest losses records up to 80 per cent (Bill *et al.,* 2014). Among various fungal pathogens, anthracnose caused by *Colletotrichummusae* is the devastating pathogen and cause up to 40 per cent of post-harvest loss (Maqbool *et al.,* 2011). The *C. musae* can able to attack the fruits before its ripening on the subcuticular region and present inside the fruit as latent infection. Later, the fruit gets ripe it may show its own typical symptoms (Vilaplana *et al.,* 2018). The typical symptoms produced by the *C. musae* may form sunken lesion on the skin of the fruits without skin bruising (Su *et al.,* 2011). To manage this disease, several chemical fungicides are being used along with cultural practices, which may include proper and safe handling of fruits, storage, transportation and use of chemicals (Krauss *et al.,* 1999; Williamson *et al.,* 2008). In general fungicides like prochloraz, imazalil and thiabendazole are used against the post-harvest pathogens of banana (Nelson *et al.,* 1983). The long term usage of the chemical fungicides may lead to development of resistance against the pathogens and may become ineffective for managing the disease and also become harmful to the consumers (Ayon-Reyna *et al.,* 2017). Due to this indiscriminate issues, several other eco-friendly management practice have been evolved and used by the producers. Among them dip

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treatment, plays a major role because of its efficiency toward the control of post-harvest pathogens. Normally dip treatment of banana fruits with hot water at 40° C can effectively control the post-harvest pathogens (Usall *et al.,* 2016). It is also an eco-friendly approach but, it affects nutritional quality, sensory properties and do not extend the shelf life of banana fruits. Some of the naturally occurring plant volatiles or the essential oils also showed to be involved in the antifungal activities by affecting the sporulation or germination of the fungal spores (Caccioni*et al.,* 1995 and Fries, 1973). Hexanal, a natural plant volatile produced by oxidative degradation of fatty acids directly contributes for the green taste of vegetables. It is an c-6 carbon aldehyde molecule is reported to effectively control the blue and grey mould of the mature apples (Sholberg and Randall, 2007). The major role of hexanal is to inhibit the enzyme activity of Phospholipase-D enzyme (PLD) which is responsible for the membrane deterioration (Paliyath and Murr, 2007).The antifungal effects on *Botrytis cinerea* and *Monilinia fructicola* on exposure to hexanal vapor and shown that the hexanal vapor reduces the decay levels on raspberry and peach fruits (Song *et al.* 2007). Similarly, the hexanal treated tomato fruits shown to have a suppressed the grey mold growth (Utto *et al.,*2008). In addition, it was reported that the pre-and post harvest application of hexanal and combination of 1-MCP was extended the shelf-life of sweet cherry. The current study was aimed to analyse the antifungal activity of Hexanal in the form of nano emulsion against the banana anthracnose and its effect on post-harvest shelf life extension of banana fruits.

# MATERIALS AND METHODS

**Cultures and virulence**

Anthracnose causing fungus, *Collectotrichummusae* were isolated from the infected banana fruit exhibiting typical anthracnose symptoms. The infected tissue portions were cut into small pieces of 1.0 to 1.5 $cm^2$, surface sterilized with 0.1 per cent mercuric chloride for 30s and washed with sterile water for three times. The infected banana tissue segments were then placed on petri plates containing molten PDA medium and incubated at room temperature (25 ± 2°C) for further fungal growth.

**Preparation of spore suspension**

The conidia of the 10 days old culture were harvested from the culture plates by adding 5 ml of sterile distilled water and rubbing the culture surface gently with sterile glass rod. The conidial suspension was then filtered with sterile muslin cloth to remove the mycelial remnants and then volume of the conidial suspension was made to final volume of 10 ml by using 0.01 per cent triton X-100 and glycerol to obtain 106 conidia $ml^{-1}$.

**Enhanced freshness formulation**

Freshly prepared hexanalnano-emulsion named Enhanced Freshness Formulation (EFF) was obtained from the Department of Nano Science and Technology, Tamil Nadu Agricultural University, Coimbatore.

**In vitro fungal mycelial growth inhibition by EFF dip treatment**

5mm diameter mycelial disc was excavated from the periphery of actively growing fungal culture. This disc was then dipped in the different concentrations of EFF (1, 2, 3, 4, 5 per cent). Then the disc was inoculated in the petri plate at room temperature. The colony growth in terms of diameter was measured daily until the control plates attained full growth of 90 mm. The inhibition of the fungal growth was calculated as per cent inhibition over control.

$$\text{Per cent inhibition over control} = \frac{\text{Control - Treatment}}{\text{Control}} \times 100$$

**Banana fruit sampling**

Banana variety Grand Naine, Cavendish group (AAA genome) was harvested from the Orchard at Tamil Nadu Agricultural University, Coimbatore. The fruits were harvested at 80 per cent maturity. The harvested fruits were transferred to the laboratory and cleaned with fresh and second wash with distilled water to remove the adherent impurities.

***In vitro* assay**

Banana hands were surface sterilized with 0.01 per cent sodium hypochlorite solution for 3 mins, then washed with clean distilled water and placed in a sterile condition. Surface sterilized banana hands were then dipped in 2 per cent EFF solution for 5 mins. Then the hands was post inoculated with the spore suspension of C. musae on the surface of the fruit skin and incubate at room temperature (25 ± 2°C).

***In vitro* disease assessment**

Per cent disease incidence (PDI) and disease severity was recorded at 4 days interval after treatment. The PDI was recorded based on the percentage of fruits get infected and the disease severity was measured by using the 1-5 score scale, whereas 1 = 0 per cent of fruit surface rotten; 2 = 1 – 25 per cent; 3 = 26 – 50 per cent; 4 = 50 – 75 per cent and 5 = 76 -100 per cent surface rotten (Sivakumar *et al*., 2002). The scoring was converted to per cent disease incidence for further interpretation (Wheeler, 1969).

$$\text{PDI} = \frac{\text{(Sum of individual rating x 100)}}{\text{Total no. of observations x Maximum score on scale}}$$

**Enzyme assays**

The fruit samples were taken from 1-2 mm (peel and pulp) and gathered at 1, 3, 6, 9 and 12 d after treatment with hexanal and control fruit. Exo- and endocarp tissue samples (0.5 g) were frozen in liquid nitrogen and stored at "80 °C. For peroxidise (PO) and phenylalanine ammonia lyase (PAL) activities, fresh samples were homogenized with 4 ml of 0.1 M sodium phosphate buffer (pH 6.4) containing 1 % polyvinylpolypyrrolidone (PVPP), centrifuged at 14,000 × g for 30 min at 4 °C and the supernatants were used as enzyme

assays. For PO (EC 1.11.1.7), the extract was allowed to react with guaiacol and the absorbance was measured at 30 s for 5 min at 460 nm (Ippolito *et al*., 2000). The PAL (EC 1.15.1.1) activity was assayed as described by Assis *et al*. (2001). Enzyme extract (1 ml) was incubated with 2 ml of borate buffer and 1 ml of L-phenylalanine (20 iM) for 30 min at 37 °C and the resultant chromophore was measured at 460 nm. All the enzyme activities were expressed as the units (U) per microgram protein. Protein concentration was determined using bovine serum albumin as a standard.

**Shelf-life studies**

Fruit samples were drawn once in three days from control and treated, till the fruit get completely spoil it. Physiological loss in weight was recorded by subtracting final weight from initial weight of the fruit and then expressed as per cent weight loss with reference to the initial weight. Total soluble solids (TSS) in fruit was measured using hand a hand held digital refractometer (ERMA, Tokyo, Japan, measurement range of 0–85 %) (Padda*et al*., 2011) and expressed in percentage.

**Statistical analysis**

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

# RESULTS AND DISCUSSION

**Isolation and morphological identification of pathogen**

The anthracnose pathogen was isolated from the infected tissues of banana and cultured on PDA medium. The pathogen initially produced whitish mycelium and turned to light brownish orange when acervulus was produced with characteristics of hyaline, septate mycelium with sickle shaped conidia. Based on the morphological characters and fast growth nature at room temperature the pathogen was confirmed as *C. musae*. The above results were supported by the findings of Ranjitham *et al*. (2011), where they had observed the significant changes in the growth of the mycelium in PDA medium and colour changed from blackish white to dark orange during the production of acervuli. Su *et al*. (2011) also reported that the *C. musae* produced white to grey aerial mycelium, conidial masses well developed with salmon orange in colour.

**EFF dip treatment on *C. musae***

The pathogen was inoculated on culture plate where media was supplemented with different concentrations of EFF. The observations showed that the mycelial growth of the fungus has totally inhibited at 2 per cent EFF at the end of 5 DAT, whereas in control the mycelium covered the entire plate (9 cm). With this result of minimum inhibitory concentration of 2 per cent EFF dip treated fruits post inoculated with *C. musae* spore suspension also showed the similar inhibitory response (Table 1). Fruits that exposed to 2 per cent EFF dip treatment had the least incidence of anthracnose as 27.3 per cent at the 12$^{th}$ day of observation. In control, the fruits remained good up to 6$^{th}$ day and the infection started thereafter and registered the highest disease incidence of 80 per cent (Table 2). Hexanal vapour at 900 il l"1 for 12 or 24 h or 450 il l"1 for 24 h was sufficient to inhibit all tested pathogens while treatment with 200 il l"1 for 24 h had only a limited effect (Song *et al*., 2007). The grey mould fungus *Botrytis cinerea*, was inhibited by a hexanal related compound (E)-2-hexenal, which differed from hexanal by having a double bond and was more effective against spores than mycelium (Fallik *et al*., 1998). Mycelial growth of C. musae was completely inhibited by chitosan concentrations of 1.0% and 1.5% during the 7-day incubation period, while at 0.5% and 0.75% concentrations, growth began on the second and third day of the incubation period, respectively (Ayon-Reyna *et al.,* 2017).

**Changes in the peroxidase (PO) and phenyl ammonialyase activities in banana fruit**

PAL is the first key enzyme involved in phenylpropanoid pathway in plant system and plays a key role in phenolics and phytoalexins biosynthesis (Bashan *et al*., 1985). An increase in the level of mRNAs encoding for PAL was observed in the early stages of interaction between bean roots and various rhizobacteria (Zdor and Anderson 1992). POs play a vital role in the regulation of plant cell elongation, phenol oxidation, cross-linking of polysaccharides, cross-linking of extension monomers, IAA oxidation, oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran *et al*., 1997). PO is associated with disease resistance in plants (Hammerschmidt and Kuc 1995) and enhanced levels of PO were noticed in fluorescent pseudomonads in plants such as sugarcane against *Colletotrichum falcatum* (Viswanathan and Samiyappan 1999), mango against *C. gloeosporioides*, chilies against *Colletotrichum capsici* (Bharathi *et al*., 2004), rose against Diplocarponrosae (Karthikeyan *et al*., 2007). Polyphenoloxidase (tyrosinase) is a copper-containing oxidase enzyme responsible for browning of fruits that mainly oxidizes phenolics to highly toxic quinines and is involved in the terminal oxidation of diseased plant tissue and role in disease resistance. PPO usually accumulates upon wounding in plants. PPO can be induced via octadecanoid defense signal pathway (Constabel *et al*., 1995).

In the present study the peroxidase activity had increased up to nine days and declined thereafter regardless of treated or control fruit. In banana fruit, treated with 2 per cent EFF and post-inoculated with *C. musae* had significantly higher peroxidase activities (1.97 changes in absorbance $min^{-1}$ $g^{-1}$ of fresh tissue) than control (0.57 changes in absorbance $min^{-1}$ $g^{-1}$ of fresh tissue) at the 9$^{th}$ day of treatment and decreased gradually thereafter. Similarly, the hexanal vapour exposed fruit had significantly higher PAL activities up to 9$^{th}$ day (0.45 nmol of transcinnamic acid $min^{-1}$ $g^{-1}$ of fresh tissue), whereas control (0.11nmol of transcinnamic

acid $min^{-1}$ $g^{-1}$ of fresh tissue) had three times lower PA(Table 3).

**Effect of 2 per cent EFF dip treatment on extension of shelf-life of banana fruits**

Physiological loss in weight (PLW) is one of the prime parameters indicating the ability of the fruits to retain its freshness during storage. The reason for weight loss during ripening is due to high energy is required to run the process; hence, starch is converted into sugar and used as energy. The exogenous application of hexanal slows down the lipoxygenases in the skin of the fruits which would have assisted in delayed ripening processes and lower PLW. During storage, the sugars tended to get accumulate in the pulp of fruits while starch declines with the progression of ripening. In the current study, the physiological loss in weight was lower in 2 per cent EFF dipped fruits (5.63 per cent) than the control fruits (6.69 per cent) at 12 days after treatment (Table 4). Total soluble solids (TSS) was measured in control and treated (2 per cent EFF) fruits and the results showed that the brix value of treated fruit was 24 whereas, in control it was 26.19 $^{O}$Brix (Table 5). As the banana starts ripening, the TSS content increases due to breakdown of starch. Since, the treated fruits are still holding the starch and it tends to indicate the extension of shelf-life which is beneficial for the long term storage and transport. It is also quite interesting to observe that pre-harvest spray of nano-emulsion of hexanal extends the shelf-life of fruits without altering the quality attributes of the fruits. exposing banana to 2 h of hexanal vapour at 1200 ppm had a shelf life of 18 d at 27 ± 2°C whereas a 4 h exposure resulted in a shelf life of only 12 d. Anusuya *et al*. (2016) reported that, a significant difference was observed in TSS content of fruits treated with hexanal vapour and without any hexanal vapour treatment. At the 9th day, the TSS content of fruits treated with 1200 ppm hexanal vapour concentration for 2 h ranged between 6.86° brix, whereas for control fruits it was found to be 13.85° brix. These results implied that hexanal vapour treatments were effective to delay breakdown of starch content, which is one of the ripening indicator for banana. Similar to this result Opiyo and Ying (2005) also reported that hexanal delayed the TSS accumulation during ripening whereas the well known ripening inhibitor 1MCP did not affect the TSS accumulation during ripening in tomato.

**Table 1. Mycelial growth of pathogen treated with EFF (cm)**

| Treatment (% EFF DIP) | Per cent inhibition over control |
|---|---|
| 1 | 22 |
| 2 | 100 |
| 3 | 100 |
| 4 | 100 |
| 5 | 100 |
| SEd | 0.25 |
| CD(0.05) | 0.58 |

**Table 2. Disease assessment**

| Days | Control | Treated |
|---|---|---|
| 3 | 22.7 | - |
| 6 | 38.6 | - |
| 9 | 55.1 | 18.2 |
| 12 | 80 | 27.3 |
| SEd | 0.99 | 0.10 |
| CD(0.05) | 2.44 | 0.25 |

**Table 3. Enzyme assays**

| Days | Peroxidase (changes in absorbance $min^{-1}$ $g^{-1}$ of fresh tissue) | | PAL (nmol of transcinnamic acid $min^{-1}$ $g^{-1}$ of fresh tissue) | |
|---|---|---|---|---|
| Control | Treated | Control | Treated | |
| 3 | 0.87 | 0.92 | 0.19 | 0.22 |
| 6 | 0.63 | 1.79 | 0.16 | 0.31 |
| 9 | 0.57 | 1.97 | 0.11 | 0.45 |
| 12 | 0.49 | 1.64 | 0.09 | 0.34 |
| SEd | 0.009 | 0.027 | 0.002 | 0.004 |
| CD(0.05) | 0.024 | 0.06 | 0.005 | 0.011 |

**Table 4. Physiological loss in weight (Per cent)**

| Days | Control | Treated |
|---|---|---|
| 3 | 0.81 | 0.94 |
| 6 | 2.24 | 3.00 |
| 9 | 5.19 | 5.32 |
| 12 | 6.69 | 5.63 |
| SEd | 0.13 | 0.02 |
| CD (0.05) | 0.33 | 0.06 |

**Table 5. Total soluble solids ($^{O}$Brix)**

| Days | Control | Treated |
|---|---|---|
| 3 | 16.28 | 16.12 |
| 6 | 18.91 | 18.44 |
| 9 | 22.43 | 21.38 |
| 12 | 26.19 | 24.00 |
| SEd | 0.007 | 0.007 |
| CD (0.05) | 0.018 | 0.18 |

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