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Production of banana bunchy top virus (BBTV)-free plantain plants by *in vitro* culture

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Banana Bunchy Top Disease (BBTD) caused by the Banana Bunchy Top Virus (BBTV) is one of the most important banana diseases in the Democratic Republic of Congo. This study focused on the production of BBTV-free plantain seedlings from infected banana plants. A total of 10 suckers from the French plantain Litete (*Musa* AAB) and the False Horn plantain Libanga Likale (*Musa* AAB) with advanced BBTD symptoms were collected. Meristematic apices excised from those suckers were cultured *in vitro* and subcultured five times. The presence of BBTV was evaluated by the Triple-Antibody Sandwich Enzyme-linked Immunosorbent Assay (TAS-ELISA). The BBTV was confirmed in all suckers prior to *in vitro* culture but 73.3% of Litete plantlets and 66.6% of Libanga Likale plantlets regenerated from meristematic tissues were virus-free. This indicates that *in vitro* culture is a simple tool to generate BBTV-free plantains.

Key words: Banana bunchy top virus (BBTV), *in vitro* tissue culture, plantains

INTRODUCTION

The Banana Bunchy Top Disease (BBTD) is one of the most devastating diseases in banana and plantain, sometimes causing 100% yield losses (Qazi, 2016). About 20 virus species belonging to 5 families have been reported to infect banana and plantain worldwide (Kumar et al., 2015). The most economically important viruses of banana are Banana Bunchy Top Virus (BBTV, genus Babuvirus, family Nanoviridae), several species of Banana Streak Viruses (BSVs, genus Badnavirus, family Caulimoviridae) and Banana Bract Mosaic Virus (BBrMV, genus Potyvirus, family Potyviridae). Of minor significance are Abaca Bunchy Top Virus (ABTV, genus Babuvirus), Abaca Mosaic Virus caused by a distinct strain of Sugarcane Mosaic Virus (SCMV) designated as SCMV-Ab (genus Potyvirus), Banana Mild Mosaic virus (BanMMV), and Banana virus X (BVX) both unassigned

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members in the family Betaflexiviridae, and Cucumber Mosaic Virus (CMV, genus Cucumovirus, family Bromoviridae). Viruses are a major concern to banana and plantain production because of their effects on yield and quality, and as constraints to the international exchange of *Musa* germplasm. Direct losses are incurred from reduced production, and indirect losses are associated with maintaining plant health, including the production of virus-free planting material. BBTV is among the top 10 viruses worldwide in terms of economic impact (Rybicki, 2015). The BBTV is transmitted by the banana aphid *Pentalonia nigronervosa* Coquerel. Its transmission efficiency is affected by temperature, the stage of life of the vector and plants during the period of collection (Anhalt et al*.,* 2008). The long-range diffusion of BBTD, however, is more closely related to the transport of infected plant material (Qazi, 2016). In sub-Saharan Africa, BBTV was first reported in the Democratic Republic of Congo (DR Congo) in the 1950s (Kumar et al*.,* 2011). It has spread throughout the country (Mukwa et al*.,* 2014). Recently, 16 BBTV isolates from the former Orientale and South Kivu provinces (North-east and central DR Congo) were compared as part of a global distribution study of BBTV, revealing a large human contribution to its dispersal over long distances (Stainton et al*.,* 2015). In DR Congo, BBTV is present in all its 11 old provinces (Kumar et al., 2011; Mukwa et al*.,* 2014; Ngama et al*.,* 2014). Farmers collect suckers from infected symptomless plants to establish new fields thereby spreading further the disease and encountering heavy yield losses. There is thus a clear need to provide farmers with virus-free planting material.

In infected plants, virus particles might be omnipresent but it is hypothesized that at least part of the meristem is virus-free. *In vitro* culture of meristems has the potential to multiply precisely these virus-free cells to amounts that allow plant regeneration from it and therefore to deliver virus-free plants. Bananas (bananas and plantains) constitute a crop that plays a major role in food security in the Democratic Republic of Congo (DR Congo). Indeed, they are rich in energy, mineral salts (potassium, calcium, phosphorus) and vitamins A, B and C. The production of bananas and plantains of DR Congo occupies the 10th position in the world. Compared with other food products, their production comes second to cassava. In addition, bananas and plantains play a role in improving the income of the population because of their high market value (Dhed'a et al., 2019).

The aim of this study is to clean plantain plants, a starchy banana of the *Musa* AAB subgroup which is widely cultivated in the Congo basin and in West Africa, from BBTV by *in vitro* culture to regenerate healthy plants free of BBTV and confirm this by TAS-ELISA.

MATERIALS AND METHODS

The plant material consisted of young suckers (30-40 cm in height) of two plantain (*Musa* AAB) varieties, 'Litete' (Figure 1) which is a French type plantain and 'Libanga Likale' (Figure 2), a False Horn type plantain.

Suckers of the two cultivars were collected around Kisangani (DR Congo) town on plants with visual symptoms of BBTD. The severity of the disease in the field was scored using a scale of 0 - 5 (0: No symptoms, 1: presence of streaks on the leaf, 2: presence of streaks on the pseudostem, 3: discoloration of the leaf keeping its normal size, 4: reduced leaf size and 5: bushy appearance at the top or Bunchy top) (Niyongere et al*.,* 2011; Ngama et al*.,* 2014). Only suckers with advanced stages of BBTV (4 and 5) were collected (Figure 2).

A total of 10 infected suckers were collected from 5 'Litete' and 5 'Libanga Likale' tufts. All suckers were tested using TAS-ELISA and were confirmed as positives. The TAS-ELISA method used involved BBTV extraction from the leaves, incubation and addition of monoclonal antibody and antibody coupled to alkaline phosphatase B in the presence of positive and negative BBTV controls. All the processes were conducted in the laboratory of the Faculty of Science of the University of Kisangani (UNIKIS).

In vitro cultures of infected plants were established on standard media with mineral salts (Murashige and Skoog, 1962) (Figure 4). This medium was enriched with 30 g/l of sucrose, 2 g/l of gelrite, nicotic acid (0.5 mg/l), pyridoxine (0.4 mg/l), thiamine (0.5 mg) and 2 mg/glycine and supplemented with a 10 µM 6-benzylaminopurine (BAP) and 1 µM of indole acetic acid (IAA) according to Banerjee et al*.* (1985, 1986) and Vuylsteke (1989) (Figure 3).

Each cultivar was subcultured 5 times at one month intervals. The *in vitro* plants were regenerated and acclimatized in the screenhouse for two months until the plantlets reached a size of 20 cm and then tested twice for BBTV by TAS-ELISA. Data were analyzed by R Software (3.1.3).

RESULTS

The two plantain cultivars were put *in vitro* and subcultured 5 times (Figure 4). There was no difference in bud proliferation between both cultivars. Indeed after the first subculture, Libanga Likale produced 7.2 proliferating buds compared to 5.2 for Litete, a non-significant difference (p-value = 0.3455; t = 1.0025). After the fifth subculture, Libanga Likale produced 8.6 proliferating buds compared to Litete which produced 11.2 proliferating buds, a non-significant difference (p-value = 0.3287; t = 1.0442).

After *in vitro* culture, the banana plants were regenerated and all the samples analyzed. Of the 30 Libanga Likale plants produced, 10 were positive and 20 negative; also, Litete produced 8 positive and 22 negative plants. The BBTV-free plants grew fast unlike plants infected with BBTV (Table 1 and Figure 5).

The results in Table 1 show that the *in vitro* culture could clean 73.3% Litete and 66.6% Libanga Likale plants. Overall, this technique cleaned 70.0% of all plants studied.

DISCUSSION

The interest of this work lies in the development of a propagation technique of healthy plants that will contribute to the improvement of the production of banana, making it possible to improve the food security in

(a) (b)

Figure 1. Cultivars used for in vitro culture (a) Litete and (b) Libanga Likale.

Level 4 Level 5

Figure 2. Typical banana bunchy top disease symptoms.

Kisangani, DR Congo.

Plantain cultivars responded quickly to *in vitro* culture as already after one subculture, the number of buds was 5.2 and 7.2 for Litete and Libanga Likale respectively. The number of proliferating buds increased by the fifth subculture with the number of buds more than doubled in Litete (11.2) while the number of buds for Libanga Likale increased less drastically (8.6). Reyes et al. (2017) found *in vitro* proliferation rates of 1.95-2.20 in plantains, while Korneva et al. (2013) found a 0.8 proliferation rate for

Figure 3. Stages of cultivation. A: Removal of all old non-meristematic tissues. B: Explant with reduced size and washed under running water. C: Disinfection successively by immersing the explant in alcohol 70% for 15 s, and the solution of calcium hypochlorite 30% for 20 min. The explant is then rinsed with sterile distilled water three times. D: Removal of the foliar tissues one after the other until extraction of the meristematic apex. E: Putting the explant in the in vitro culture medium. F: Transfer of tubes containing the explants into the culture chamber. G: Proliferation of the buds after a minimum of two weeks. H: Subculture (separation of buds and their transfer to a new medium). I: Regeneration of buds to rooted seedlings. J and K: Transfer of vitro plants to pots.

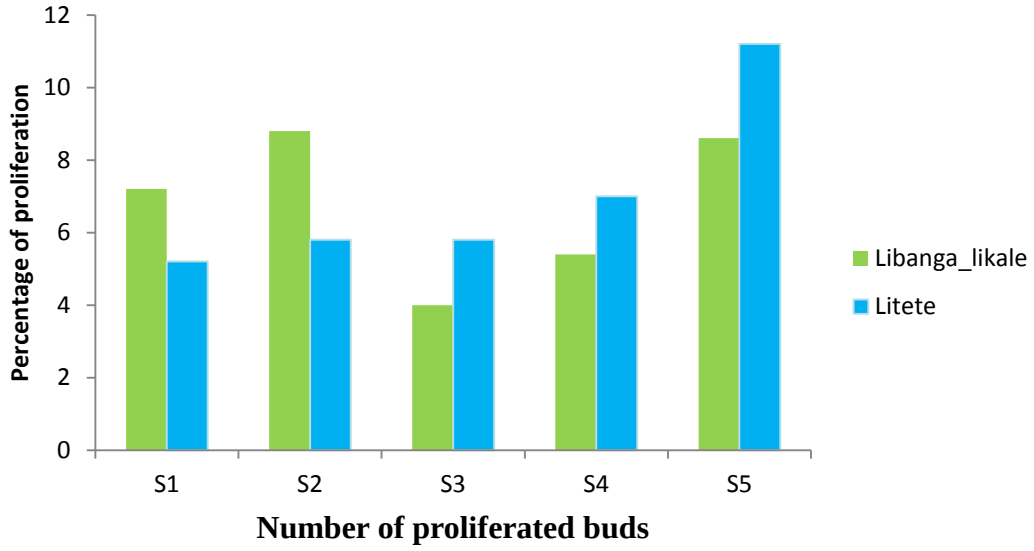

Figure 4. Number of buds produced in vitro during 5 subcultures (S1- S5) of the two plantain cultivars, Litete and Libanga Likale.

Table 1. Health status of Libanga Likale and Litete after *in vitro* culture.

Cultivars	Plants tested	Positive plants	Negative plants	Remediation rate (%)
Libanga Likale	30	10	20	66.6
Litete	30	8	22	73.3
Total	60	18	42	70.0

Figure 5. Banana bunchy top virus-free plantlet (left), and infected plantlet (right).

plantain and 1.86 for banana. Our results are also in line with Dhed'a (1992), who showed that 10 µM BAP increased the proliferation rates in *in vitro* with 3.5 for the plantain cultivar Three Hand Planty (*Musa* AAB) with 18.6, 12.4 and 21.4 for the cooking banana cultivars Bluggoe, Cardaba and Saba (*Musa* ABB) and 7.1 for the dessert banana cutivar Yangambi Km5 (*Musa* AAA). Roels et al. (2005) obtained a proliferation rate of 3-5 in the dessert Cavendish (AAA) subgroup. The virus detection by TAS-ELISA showed that 73.3% of Litete and 66.6% of Libanga Likale were found to be BBTV free after plant regeneration. Our results are in line with Morel and Martin (1952), who by taking meristematic spikes of dalhias obtained dahlias free from the mosaic of dalhias and the spotted wilt virus which are caused by RNA viruses. On the other hand, it is by using the meristem culture that Wang and Hu (1980) managed to eliminate more than 70 known diseases in more than 40 different species. Sweet et al. (1979) obtained a high level of purification from the "Nepo" viruses (RRV = Raspberry ringspot, AMV = Arabis mosaic virus) by coupling thermotherapy and meristem culture, whereas meristem culture alone was sufficient to eliminate cucumber mosaic (CMV). Mosella et al. (1980) obtained 57% plants free from N.R.S.V (Sharka necrotic ringspot virus) and 72% for Sharka starting with 0.4 -0.8 mm explants. Panis et al. (2001) also found that 37.9% of banana and plantain plantlets regenerated from cryopreserved proliferation meristems tested negative for ELISA. However, since possible remediation mechanisms are not fully

understood, the most likely assumptions are: - absence of vascular connections between the meristem and the underlying tissues; viruses must progress symplastically or apoplastically rather than vascularly to reach the meristem, which is slower; an actively growing meristem can therefore "escape" the viruses,- absence of plasmodesmes at the meristem, hence slowing propagation symplastically, intense competition between meristematic cells under active division and viral particles for nucleoproteins,- presence of inhibiting substances,- in case of excision of the meristem, temporary unavailability of enzymes necessary for viral replication; this unavailability is a function of the size of the meristem, and is therefore longer as the meristem is small. It has been observed that small meristems that contain viruses can regenerate healthy plants. Our results on sanitation show that it is possible to obtain a high level of plants sanitized by in vitro culture.

In vitro culture of BBTV infected plantain cultivars is a simple tool to obtain virus free clean planting material from plants with advanced symptoms. With only five subcultures, we obtained 73.3% virus-free plants in the Litete cultivar and 66.6% in the cultivar Libanga Likale. Hence it is hypothized that more virus-free plants can be obtained in either cultivar by either increasing the number of subcultures or increasing the concentration of BAP, as Dhed'a et al. (1991) showed that 100 µM BAP increases drastically the proliferation rate. Since the proliferation rate varies a lot between different banana cultivars with a different genomic background, we also speculate that the rates of cultivars becoming virus-free also vary within the banana subgroup.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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