Impact Of Thermotherapy And Chlorothalonil On Plantlets Production Of Some Genotypes Of Cassava (Manihot Esculenta Crantz) Produce In Benin

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ABSTRACT: Cassava (Manihot esculenta) is a starchy root plant of great economic importance in sub-Saharan Africa and particularly in Benin. Its production is confronted to virus diseases which cause a considerable losses of yield. This work aims to determine the impact of thermotherapy and chlorothalonil in the production of cassava material of plantation. Cuttings of four varieties: RB89509, BEN86052, 91/02319, 92B/0057 are cultivated under two conditions of thermotherapy and a control under greenhouse during 4 weeks. These different conditions are: a closed drying oven with 16 hours photoperiod at 40 °C the day and 36°C the night; a drying oven "Binder" with photoperiod of 12 hours at 38°C the day and 28°C the night and the control carried out under the conditions of the greenhouse. The media used was Murashige and Skoog (MS) added with various amounts of chlorothalonil: 0.6 g/l and 2g/l and control without chlorothalonil. Both techniques of thermotherapy eliminate the virus symptoms of cassava at the rate of 0 seedling infected in thermotherapy against 16 seedlings in natural condition. The technique of closed drying oven significantly favors the production of nodes at 5% level (p=0.000) and shoots (p=0.02) on the other hand Binder drying oven has no significant effect on the production of shoots (p=0.68). The chlorothalonil had a positive effect on in vitro infestations elimination of cassava (p<0.05) but influenced the growth and development of cassava explants by reducing of nodes production (p<0.01) without a lethal effect on the plantlets until the dose of 2g/l.

Keywords: Manihot esculenta, In vitro culture, Thermotherapy, chlorothalonil, Benin

1- BACKGROUND

Cassava (*Manihot esculenta* Crantz) is an important tropical food crop in the world [1]. In Africa, an estimated 70 million of people whose diet depends to cassava. This is their main commodity and contributes to their feeding at 500 kcal per day per person [2]. In Benin, cassava constitute the third sensitive products to food security and famer incomes. It is therefore one of the major products in the creation of national wealth [3]. Cassava production is severely harmed by diseases and pests especially the cassava mosaic disease (CMD) [4]. These diseases cause enormous damages that significantly reduce their performance. Yield losses causing by the disease are of the order of 20 to 80% of the production [5]. The mosaic has become a persistent and significant threat to cassava production and food security of the population [4]. It is caused by a virus that is transmitted by whitefly (*Bemesia tabaci*) which is an insect vector.

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This raises a real problem of availability and production of healthy cassava planting material. Recent literature have reported cases of using *in vitro* tissue culture in the production of cassava plants free to viruses [6], [7], [8]. In the aim to improve the tissue culture results, it was developed thermotherapy techniques that can reduce the viral load of the mother plants before sampling the explants. Thermotherapy is an effective method in this approach. This technique consists in culturing at high temperature cuttings [8]. Some use light sources to produce heat and other ovens [9]. Others also use hot water as a heat source [10]. Despite strict precautions of tissue culture manipulation, the explants dearly acquired by thermotherapy is subject to multiple infections, which justifies the use of chlorothalonil, an effective disinfectant [11],[12] in the media. Unfortunately, the studies have not been cases of the impact of this fungicide on the growth of plantlets. This work aims to analyze the contribution of thermotherapy and chlorothalonil in the availability of healthy cassava plantlets as seeds.

2- MATERIALS AND METHODS

The experiments were carried on four improved cassava varieties (RB89509, BEN86052, 91/02319, 92B/0057) from South Agricultural Research Centre (ARC-South) of National Institute of Agricultural Research of Benin (INRAB). The cuttings were collected on older than 8 months plants severely expressing mosaic symptoms.

2.1 Different conditions for obtaining mother plants

After disinfection with methyltiophanate 70% for 10 minutes cuttings are placed in polyethylene pots filled with soil treated with carbofuran. Three conditions of production mother plants including two in thermotherapy and a witness were used. For the control, the cuttings were placed in greenhouses at room temperature with a 12h photoperiod (Fig.1.a). As for thermotherapy; the first was to place the pots containing the cassava cuttings in a closed oven at a

temperature of 36 °C for 8 hours of darkness and 40 °C for 16 hours light [13] (Fig.1.b). The plants were watered regularly to maintain a favorable humidity for their development. The oven was composed of close bulbs producing 260 watts giving a brightness of 8000 lux, a ventilation system to homogenize the internal temperature, a thermometer for temperature measurement and a thermostat to keep the temperature. The second condition of thermotherapy was to seal the ends of cassava cuttings with parafilm and place them in an oven (Binder) at 28 °C for 12 hours of darkness and 38 °C for 12h brightness [9] (Fig.1.c). The lamp used in this technique had only played the role of light source.

2.2 Tissue culture medium

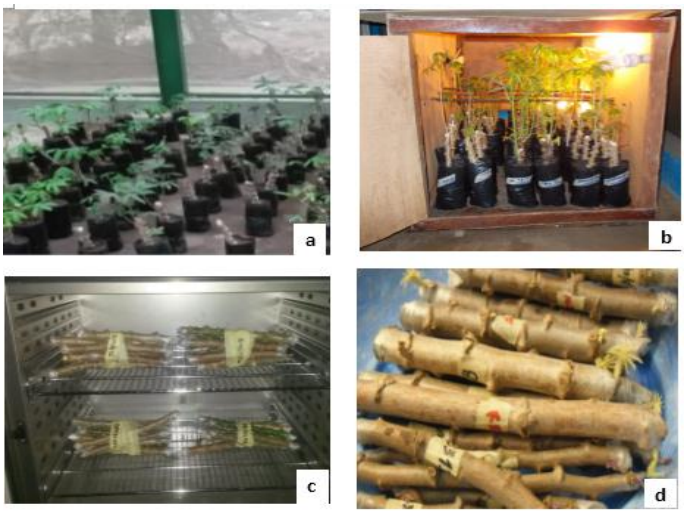
The basic Murashige and Skoog (MS) medium supplemented with 100 µl of NAA and 500µl of BAP in the concentration of 0.1g/ml in one liter of medium was used. The mediums were different in the concentration of chlorothalonil used: 0.6g/L; 2 g/L and a control (no chlorothalonil). The mixture of nutrients brought to the agitation on a magnetic stirrer without heating for 5 to 10 minutes. During stirring, poured the amount of chlorothalonil and adjusting the pH of the mixture to 5.8 with NaOH (base) or HCl (acid), while stirring the solution. This solution was mixed with an agar solution prepared beforehand. The mixture was heated to boiling on a hot plate with continuous agitation. The prepared medium was then dispensed into test tubes with 10 ml of medium per tube. The tubes are sealed with the previously sterilized cotton wool. The sterilization of the medium takes place in an autoclave at 120 °C for 15 minutes under a pressure of 1.5 bar.

2.3 Initiation *in vitro* cassava explants

The explants were taken from young shoots obtained under different conditions of thermotherapy. The explants were immersed in 70° ethanol for 5 minutes and then in 10% of sodium hypochlorite solution to which was added a few drops of Tween 20 for 20 minutes under a horizontal laminar flow. They were then subjected to three successive rinses with sterile distilled water. The obtaining explants were seeded on three different mediums and cultured in the culture room at a temperature of 27 ± 1 °C, photoperiod 12 hours with a relative humidity of the room that is 80%

2.4 Experimental design and data analysis

20 cuttings of each variety were considered in each experimental condition with three repetitions. Multivariate analysis was carried out to compare the effects of different treatments of thermotherapy on the absence or not of symptoms, shoot production and output nodes. The model with interaction between variables was suitable for analyzing the effect of processing on the number of nodes, while the model without interaction between variables is suitable for analyzing the effect of processing on the number of shoots and the absence or not of viral symptoms. To evaluate the influence of chlorothalonil on infection *in vitro* explants of different varieties of cassava, 36 explants initiated cultured *in vitro* with three treatment with three replicates. The regression tests were performed to determine the influence of chlorothalonil on the infestation rate of plantlets regenerated. All these analyzes were performed using the R software



***Legend: a** - greenhouse cassava cuttings; **b** - thermotherapy design P1-T1 (16 h / 8 h 40°C / 36 °C); **c**- thermotherapy design P2-T2 (12 h / 12 h-38 °C / 28 °C); **d**- Cassava cuttings germinated*

***Fig.1** Different conditions of mother plants production.*

3- RESULTS

3.1 Influence of thermotherapy on symptoms of cassava mosaic

The various techniques of thermotherapy strongly influence the symptoms of cassava mosaic in different varieties. Indeed, whatever the variety, no symptoms of cassava mosaic was observed while the number of infected plants per varieties grown under greenhouse conditions varies from 7 to 16. The infestation rate varied between 35 % and 80% of the plants grown (Fig.2). Therefore, both thermotherapy techniques are very effective in reducing the viral load in cassava plants.

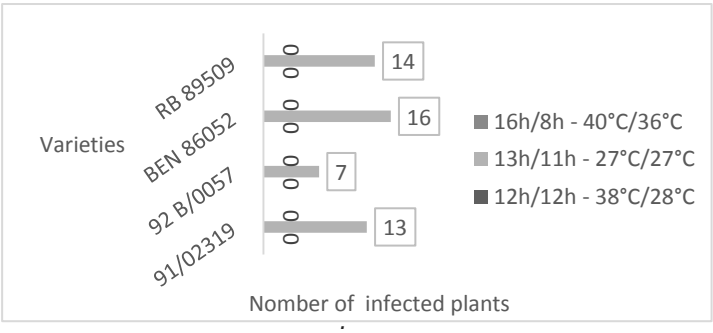


***Fig. 2.** Histogram showing the influence of thermotherapy on cassava mosaic symptoms.*

The level of symptoms decreased significantly in the variety 92B / 0057. Indeed, there was a strong decrease in symptoms, or precisely 21.28 units according to P1-T1 and P2-T2 treatments. Values equivalent probabilities (0.98) indicate that there was no significant difference between the effects of the two treatments of thermotherapy (Fig.2.).

3.2 Impact of thermotherapy techniques on nodes production

The two techniques of thermotherapy had not the same effects on output nodes of the plants of different varieties.

Indeed, the technique P1-T1 (16h/8h - 40°C/36°C) favors more production nodes than the technique P2-T2 (12h/12h - 38 °C/28 °C) (Fig 3). The analysis was showed that the heat has a very significant effect ($p < 0.05$) the number of nodes produced by seedlings.

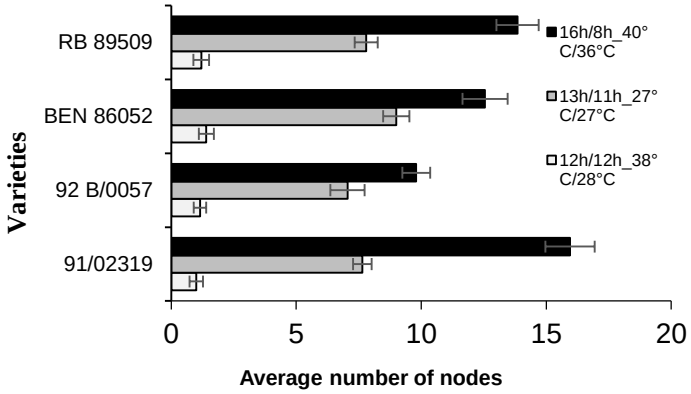


Fig. 3. *Variation in average number of nodes per varieties of different treatments.*

Applying the treatment P1-T1 (16h/8h - 40 °C/36 °C) compared with the control which was the plants produced in greenhouses, an increase of 0.7 unit nodes was noted in all varieties while this number decreases to 2.03 units by applying treatment P2-T2 (12 h/12h -38 °C/28 °C). Furthermore, the interaction between the treatment P1-T1 (16 h / 8 h-40 ° C / 36 ° C) and varieties has a significant effect ($p = 0.004$) and regarding the third negative range of 0,4 units of nodes in seedlings of varieties 92B/0057 and BEN 86052

3.3 Influence of chlorothalonil on the *in vitro* cultivation of cassava plants

3.3.1 Effect of chlorothalonil on the infestation rate *in vitro* of cassava plants

Chlorothalonil acts on infestation of cassava plantlets regenerated. In the absence of chlorothalonil the infestation rate of regenerated plantlets is high (Table1) but when the dose of chlorothalonil was increasingly added in the medium, there was a drastic decreasing of infections rate in the plantlets reaching zero (0) when the dose of chlorothalonil was 2 g/L in the medium (Table 1). Photographs of uninfected plantlets and the infected plantlets were presented in Figure 4.

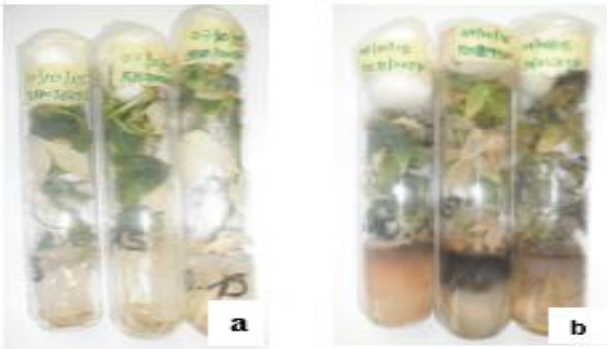


Fig.4. ***a-*** *Uninfected plantlets on medium supplemented with 2g/L of chlorothalonil;* ***b-*** *Infected plantlets on medium without chlorothalonil*

Infestation rate of plantlets decreases as the dose of chlorothalonil as increases in the medium. Increasing the dose of 0 to 2 g/L causes a decrease in infection rate of 1.15 unit (Table 1). The negative sign of the coefficients indicates an inverse relationship between the rate of infestation and the different doses applied.

Table 1*: Regression Parameters related to the dose and rate of infestation*

	Estimate	Pr (>|t|)	Signif. codes
(Intercept)	-0.5814	0.0602	-
Doses (0.6)	-0.2970	0.3695	-
Doses (2)	-1.1453	**0.0193**	**
BEN: 86052	-0.6972	0.1164	-
91/02319	-0.4823	0.2409	-
92 B/0057	-0.4823	0.2409	-
RB 89509	-0.6865	0.1065	-

*** Very significant, ** Significant , * few significant , - Not meaningful

3.3.2 Effect of Chlorothalonil on regeneration *in vitro* cassava

The box plot in Figure 5 shows the number of nodes based on chlorothalonil doses for all varieties. When the dose of chlorothalonil is 0g/L, the average number of nodes of plantlets was 5. On the other hand, adding 0.6g/L and 2g/L in the medium is passed respectively average number of nodes 3 and 2 (Fig. 5.).

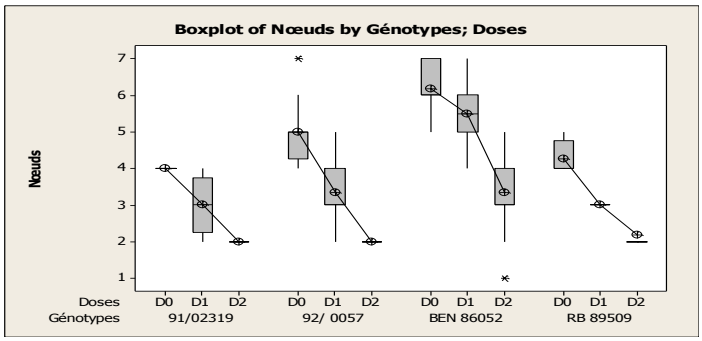


Fig.5. *Box plot related to the influence of chlorothalonil on output nodes in each variety.*

Chlorothalonil had a significant negative effect on the number of nodes. Its addition to the medium causes a decrease in the number of nodes. The different varieties react differently in the presence of chlorothalonil. A variety BEN 86052 produced more nodes in the presence of chlorothalonil against the variety 91/02319 which produces fewer nodes.

4- DISCUSSION

4-1. Heat effect on cassava mosaic symptoms

The different techniques of thermotherapy eliminate cassava mosaic symptoms. Thermotherapy was a heat treatment, whatever the variety, lack visual symptoms of virus diseases was noted on plants that have undergone this treatment. A similar results have been obtained by Wasswa *et al*. [13] who showed that thermotherapy is very effective for the

elimination of symptoms of viral infection on some cassava varieties because the high temperature destroys the chemical processes in the life cycle of the virus. The number of plants showing visual symptoms of the mosaic greenhouse varies from 7 (37%) to 16 (80%) infected plants. These results are consistent with those obtained by Cacai et al. [9] who showed in their study that thermotherapy revealed a total visual symptoms of virus disease on cassava plants lack cultured at an elevated temperature. These results stem from the lack of virus replication in the actively dividing virus [14]. The biochemical process that explains these results has been elucidated by Allam, [15], who in his work has shown that under high temperature, protein subunits that protect the nucleic acid of the virus become weak and have cracks, allowing the attack of the virus by nucleases.

4.2 Heat effect on cassava nodes production

The high average nodes obtained from all cassava plants from cuttings subjected to thermotherapy compared with averages in greenhouse confirm the stimulatory effect of thermotherapy on growing cassava plant stems [8]. Indeed, applying the P1-T1 treatment increase of 0.7 node unit among all varieties while this number decreased to 2.03 nodes units by applying processing P2-T2. This observation is due to the fact that the cuttings of different varieties are grown on a substrate (potting soil) in the first thermotherapy treatment (40°C/36°C). These results were in agreement with those obtained by Cacai et al. [8] which showed that the thermotherapy treatment in closed oven promotes much more production nodes in different cassava varieties. Furthermore, the interaction between the P1-T1 and treatment varieties has a significant negative effect of 0.4 units of the average number of nodes in plants varieties 92B/0057 and BEN86052. Thus, varieties 92B/0057 and BEN86052 have lower capacity of production of nodes in the thermotherapy than 91/02319 and RB89509 varieties. This is related to genotypes of 92B/0057 and BEN86052 varieties that produces fewer nodes under high temperature of P1-T1 treatment.

4.3 Influence of chlorothalonil on plantlets production

The addition of 2g/L chlorothalonil to the medium caused the decreasing of infection rates to 1.15 unit. Chlorothalonil inhibits the development of microorganisms in the medium. Like the mercuric chloride and sodium hypochlorite, chlorothalonil acted positively on the elimination of infestations *in vitro*. These results confirm the work of Chen et *al*. [16], Watt et al, [12]. Lather and Jansen [17] which showed that chlorothalonil fight effectively against the growth of microorganisms *in vitro* culture and in the medium [11], [18]. The antifungal effect of this product was highlighted on 6 mildew (*Puccinia hemerocallidis, P. iridis, P. menthae, P. sorrel, P. pelargonii-zonalis and Pucciniastrum vaccinii*) [19]. Moreover, chlorothalonil influences the growth and development of cassava plantlets without causing the death of tissue culture plants. Indeed, the correlation between the dose and the number of nodes is 0.66. Despite its growth slackening effect, chlorothalonil is not lethal to cassava plantlets. Chlorothalonil could prevent the regeneration of cassava explants in a dose higher than 2g /L in the medium. Production by plantlets nodes differs from one variety to another regardless of the dose used chlorothalonil. This difference observed *in vitro* plants of different varieties would be related to the factor "genotype" of the plant. The work is original and it assesses the impact of this on the *in vitro* cultivation of cassava.

5- CONCLUSION

This work which is part of a production of plantlets free to virus leads to the following conclusion. It helps to have techniques that can guarantee the production of mother plants without visual symptoms of virus diseases and considerable explants through different modalities of thermotherapy with more production nodes in condition (40 °C /36 °C) that met by the condition (38°C/28°C). Moreover, chlorothalonil acts positively on eliminating of plantlets infestations. Nevertheless, it has an effect on the regeneration slowing nodes formation of plantlets without being lethal to the limit dose used in our experiment.

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REFERENCES

[1] Liu, J., Zheng, Q., Ma. Q,, Gadidasu, K.K., Zhang, P., 2011**.** Cassava Genetic Transformation and its Application in Breeding. Journal of Integrative Plant Biology 53 (7): 552–569.

[2] FAO, 2010. Les maladies du manioc en Afrique centrale, orientale et australe (CaCESA) centrale, orientale et australe (CaCESA). Cadre de gestion des crises pour la filière alimentaire. ISBN 978-92-5-106379-8.

[3] Bio G.S., 2011. Conférence des Nations Unies sur pays les moins avancés; 27p.

[4] Thresh J.M., Otim –Nape, G.W., Legg, J.P., Fagette D., 1997. African cassava mosaic virus disease: the magnitude of the problem. African Journal of Root and Tuber Crops 2 (1&2): pp. 13-19.

[5] Givord, L., Fargette, D., Kounounguissa, B., Thouvenel, J.C., Walter, B., Van, R., 1994. Detection of geminivirus from tropical countries by a double monoclonal antibody ELISA using antibodies to African cassava mosaic virus. Agronomie 14, 327-333.

[6] Ahanhanzo C., Agbangla C., Agassounon D.T.M., Cacaï G.H.T., Dramane K., 2008. Etude comparative de l'influence des régulateurs de croissance sur la morphogénèse (in vitro) de quelques variétés de Manihot esculenta Crantz (manioc-Euphorbiaceae) du Bénin. Rev. CAMES - Série A, 07: 47-52.

[7] Cacaï G. H. T., Ahanhanzo C., Dangou J.S., Houedjissin S.S., Agbangla C., 2012. Effets de différentes combinaisons hormonales sur l'organogenèse in vitro de quelques cultivars locaux et

variétés améliorées de Manihot esculenta Crantz (manioc-Euphorbiaceae) cultivées au Bénin. Int. J. Biol. Chem. Sci. 6(4) : 1593-1607.

[8] Cacaï, G.H.T., Adoukonou-Sagbadja, H., Kumulugui, B. S., Ondo, O.P., Houngue, J., Ahanhanzo C., 2013. Eradication of cassava (Manihot esculenta) mosaic symptoms through thermotherapy and meristem cultured in vitro. International Journal of Agronomy and Plant Production, vol 4(S), 3697-3701.

[9] IITA, 2007. Cassava in vitro processing and gene banking. IITA Genebank series 2007.

[10] Zinga, I., Chiroleu, F. Legg, J., Lefeuvre, P, Komba, E. K., Semballa, S. Yandia, S. P., Mandakombo, N. B., Reynaud, B., Lett, J.M. 2012. Epidemiological assessment of cassava mosaic disease in Central African Republic reveals the importance of mixed viral infection and poor health of plant cuttings. Crop Protection 44 (2013) 6e12

[11] Colombo, L.A., Faria, R.T, de Carvalho, J.F.R.P., de Assis, A.M., de Fonseca, I.C., 2004. The influence of the fungicide chlorothalonil on the vegetative and root development in vitro of two Brazilian orchid species. Acta Scientiarum - Agronomy Vol. 26 No. 2 pp. 253-258.

[12] Watt, M.P., Gauntlett, B.A., Blakeway, F.C., 2010. Effect of Anti-Fungal Agents on In Vitro Cultures of Eucalyptus grandis South African Forestry Journal Vol. 175 pp 23-27.

[13] Wasswa, P., Alicai, T., Mukasa, S.B., 2010. Optimization of in vitro techniques for Cassava brown streak virus elimination from infected cassava clones. African Crop Science Journal, 18: 235 – 241.

[14] Valentine, T.A., Roberts, I.M., Oparka, K.J., 2001. Inhibition of tobacco mosaic virus replication in lateral roots is dependent on an activated meristem-derived signal. Unit of Cell Biology 219: pp. 184-196.

[15] Allam E. K., 2000. Eradication of Banana bunchy top virus and Banana mosaic virus from diseased banana plants. Annals of Agriculture Science 45:33- 48.

[16] Chen, S.K., Edwards C.A., Subler, S., 2001. Effects of the fungicides benomyl, captan and chlorothalonil on soil microbial activity and nitrogen dynamics in laboratory incubations. Soil Biol Biochem 33: 1971-1980.

[17] Latteur, G., Jansen, J.P., 2002. Effects of 20 fungicides on the infectivity of conidia of the aphid entomopathogenic fungus Erynia neoaphidis. BioControl 47: pp. 435-444.

[18] Habte M. and Manjunath, A. 1992. External and internal P requirements of plant species differing in their mycorrhizal dependency. Arid Soil Res. Rehab. 6:271-284.

[19] Mueller, D.S., Jeffers, S.N., Buck, J.W., 2005. Toxicity of fungicides to urediniospores of six rust fungi that occur on ornamental crops. Plant Dis 89: pp. 255-261