

# EVALUATION OF CULTURING METHODS FOR *Scutellonema bradys* IN YAMS

## ABSTRACT

The study evaluated eight culturing methods for *S. bradys*. culturing using in-vitro yam plantlets (tissue culture), kenaf callus, yam disc inoculated the same day, yam disc inoculated five days after disc preparation, yam disc placed on water agar, whole yam tuber, and yam setts planted in potted soil (control). Each method was laid in a completely randomised design with five replicates and inoculated with fifty individuals of *S. bradys*. After eight weeks of incubation, *S. bradys* were extracted from media, plant roots and tubers collected from each method. Final population was estimated and reproductive factor (RF) was calculated. The highest RF value was recorded in whole yam tuber method and tissue culture method with no significance differences ( $p = .05$ ). *S. bradys* did not survive in the yam disc method evaluated. The findings from the study show that whole the yam tuber method as the best option for culturing *S. bradys* while the use of tissue culture plantlets may hold some promise.

Key words: *Scutellonema bradys*, Inoculum, population, *Scutellonema bradys*, tissue culture

## INTRODUCTION

Cultivated yam belongs to the family *Dioscoreaceae* and to the genus *Dioscorea* L. [1]. This genus is reported to comprise 600 species [2]. The most important edible yam known is believed to have originated in the tropical areas of South East Asia, South America and Africa [3]. Yam is the second most important root and tuber crop in the Africa and contributes more than 200 calories daily to 60 million people, particularly in the yam belt [4]. It is a valuable source of carbohydrate in the diet of West Africans and parts of South East Asia, India, Islands of the South Pacific, the Caribbean and part of Brazil [5]. Yam is becoming increasingly important as the major source of saprogenic precursors of cortisone [5]. It is composed mainly of starch, with a small amount of proteins, lipids [6], all the vitamin C required by consumers [7], and the crop is rich in minerals [6].

31 Yam production is adversely affected by several factors, which include limited availability  
32 and cost of planting material, high cost of labour for operations such as land preparation,  
33 staking, weeding, harvesting, storage, pests and diseases [8]. Yam is prone to infection from  
34 the seedling stage through to harvesting and even in storage [9]. Reported that post harvest  
35 losses of tubers could be as high as 30% in storage [10]. 20% of post-harvest losses of yam in  
36 storage are due to diseases, to a great extent, parasitic attacks directly reflect the biocoenosis  
37 of the soil, in that they often include a complex of bacteria, fungi, nematodes and insects [11].

38 Among the nematodes associated with yam cultivation, three are considered to be major  
39 constraints namely root-knot nematodes (*Meloidogyne* spp.), the lesion nematode  
40 (*Pratylenchus coffeae*) and the yam nematode (*Scutellonema bradys*) [12]. The yam  
41 nematode, *Scutellonema bradys*, is found in the rhizosphere of soil around yam (*Dioscorea*  
42 spp.) but mainly occurs as a migratory endoparasite of roots and tubers [13]. In the tuber, the  
43 nematode is primarily confined to the sub-dermal, peridermal and underlying  
44 parenchymatous tissues in the outer 1 to 2 cm of tubers [14] where it feeds intracellularly in  
45 rupturing of cell walls and forming of cavities [13]. It continues to feed and reproduce in  
46 yam tubers stored after harvesting [13][14]. *S. bradys* reproduces and builds up large  
47 populations in stored yam tubers and causes severe damage during storage [15].

48 Yams, when grown mainly as a subsistence crop. Farmers have largely relied on natural  
49 variation for their selection of suitable varieties of yam to cope with the damage caused by  
50 plant-parasitic nematode rather than use of pesticides. Nematode resistant yam cultivars can  
51 be one of the most useful, economical and effective means of managing nematodes for  
52 resource-poor farmers. The process of identifying resistance involves screening of existing  
53 and developing germplasm. Hence the need for a screening protocol that is highly efficient,  
54 time saving and economical. When screening for resistance, or undertaking routine studies  
55 with a nematode pest, an important precursor involves a readily available and consistent  
56 supply of the nematode inoculum. Currently no suitable artificial media or culturing  
57 mechanism has been identified for the culturing and production of *S. bradys*. Though  
58 nematode species can be artificially cultured on various media such as carrot discs, callus  
59 tissue, tomato roots [16], the only documented method that has provided successful  
60 multiplication of *S. bradys* was found by Kwoseh [17], who recorded an increase of 240 fold  
61 five months after inoculating yam tuber slices with 30 nematodes.

62 Infected tubers are not always available during the whole year and can also be difficult to  
 63 maintain for nematode multiplication. Moreover, yam tuber deterioration due to invasion by  
 64 fungi, bacteria and mites after nematode infection reduces nematode populations on the  
 65 tubers [18]. It would therefore be useful to have a continuous supply of nematodes for  
 66 experiments during those periods of the year when *S. bradys*-infected tubers will not be  
 67 available and alternatively provide a pure source of inoculum throughout the year. This was  
 68 an objective of Kwoseh [17], who assessed the multiplication of *S. bradys* on yam tuber  
 69 slices, but failed to identify a suitable artificial medium.  as the objective of this study,  
 70 therefore, to evaluate various culturing methods; using in-vitro yam plantlet (tissue culture),  
 71 kenaf callus, yam disc inoculated the same day, yam disc inoculated five days after disc  
 72 preparation, yam disc placed on water agar, whole yam tuber, and yam sett planted in potted  
 73 soil, for *Scutellonema bradys* in yam.

## 74 **Materials and Methods**

### 75 **Source of yam tubers**

76 Healthy yam tubers were collected from the yam barn of International Institute of Tropical  
 77 Agriculture (IITA) Ibadan. Yam tubers showing ptoms of *Scutellonema bradys* infection  
 78 were also obtained from IITA Ibadan and Bodija market

### 79 **Extraction of *Scutellonema bradys***

80 Nematodes were extracted, from infected yam tubers showing symptoms of dry rot disease,  
 81 using the extraction tray method, according to Coyne *et al* [19]. The part of the yam showing  
 82 symptoms of dry rot was peeled and then chopped 3 - 5 mm pieces and placed in the  
 83 extraction set-up. The chopped diseased yam were then evenly distributed on the tissue.  
 84 Water was added to the set up through the area between the sieve and the plate. The set up  
 85 was left for 72 hours, with daily collection of extracted nematodes and addition of more  
 86 water. The extracts were all poured into a large 2-l beaker, then later concentrated by sieving  
 87 (38µm). The concentrated nematodes were rinsed out into a 250 ml capacity beaker

### 88 **Estimation of nematode population in inoculum**

89 In order to get 50 *S. bradys* per 1 ml, 10 ml of the extract was taken out at each time  
 90 dispensed into a separate beaker. A syringe was used to draw 1 ml of the 10 ml extract and

transferred into a counting slide and counted. Water was then added to the beaker to dilute the extract to give a concentration of about 50 individuals of *S. bradys* per ml.

### **Sterilization**

The glass-ware were sterilized in the oven at 160°C for 2 hours. Scalpels and cock borers were autoclaved at 121°C for 15 min before use, and were immersed into 70% alcohol then flamed between use. The working surface was wiped with 70% alcohol. Soil was sterilised at 120°C for two hours in the soil sterilizing chamber of the Department of Crop Protection and Environmental Biology, University of Ibadan

The *S. bradys* inoculum to be sterilized was poured in a suspension on to a 38 µm sieve from the beaker. The sieve containing nematodes was immersed into a 150 mm -dish containing 0.01% mercury chloride for 2 min and rinsed in three changes of sterile distilled water. The nematodes were then rinsed out of the sieve into a sterile test tube with sterile distilled water. Streptomycin sulphate solution (6000 Mg/L) was prepared and added to the nematode suspension at a ratio of 1 (streptomycin) :2 (sterile distilled water) [20]. This was left to stand overnight. The supernatant was siphoned off with a sterile pipette, leaving the *S. bradys* at the base of the test tube and replaced with sterile distilled water.

### **Evaluation of culturing methods**

#### **Yam disc method**

Yam tubers were washed thoroughly with soap and rinsed thoroughly with distilled water, then surfaced sterilised by rinsing in 1% sodium hypochlorite. A sterile knife was used to cut 1-2 cm off both ends of the yam tuber. Tubers were then peeled, and cut into 2 cm diameter discs into petri dishes under the lamina flow chamber. The knife was dipped inside ethanol and flamed before each contact. Each yam disc was surface sterilized by dipping it in 10% sodium hypochlorite, then dipping in 70% alcohol and flaming slightly. The yam discs were thereafter placed in  sterile petri dishes [20].

The yam disc method procedure was varied to give three different yam disc methods. In the first yam disc method, the yam discs were inoculated with *Scutellonema bradys* immediately after preparation. For the second yam disc method, the yam discs were allowed a period of incubation of  days before inoculation. In the third method, yam discs were placed in agar then inoculated.

50 individuals of *S. bradys* were inoculated on each sterile yam disc under the lamina flow hood, and the petri dishes were sealed with parafilm. The inoculated yam discs were kept at room temperature for 60 days, after which the nematodes were extracted for population estimation using the extraction tray method. After extraction, the nematode suspension was concentrated to 50 ml, *Scutellonema bradys* population was counted from 2 ml from the homogenized nematode suspension, in a counting dish. Three representative samples were taken from the suspension for counting and the mean value was calculated. This average value was used to estimate the total nematode population extracted per yam disc. Reproductive factor (RF) was calculated using the formular  $R = pf/pi$ , where pf is the final population and pi is the initial population. This was done for each of the experiments.

### **Whole yam tuber method**

Yam tubers were washed thoroughly with soap and rinsed properly with distilled water, then surfaced sterilized by rinsing in 1% Sodium hypochlorite. The nematode inoculum was sterilized using the method of Cruz *et al* [20] as previously described. A sterile cork borer was used to bore three holes of about 10 mm depth and 5 mm diameter on the tuber and the discs were placed in a sterile petri dish, this was used to cover the hole after inoculation with nematodes.

Each hole was inoculated with 50 individuals of *S. bradys* using a sterile syringe. The water was allowed to be absorbed by the tuber, the hole was plugged by the disc removed and then sealed with parafilm. The tubers were labelled, to include cultivars name and date of inoculation. They were maintained at room temperature in the laboratory for two months. The experiment was arranged in a complete randomised design and replicated five times. The inoculated yam tubers were kept at room temperature for 60 days, after which the nematodes were extracted for population estimation, using the extraction tray method.

After extraction, the nematode suspension was concentrated to 50 ml, from which nematodes were assessed as previously described. Following this total number of nematodes and RF were computed as previously described.

### **Kenaf callus as a medium for culturing nematode**

Seeds of kenaf, NHC 15 variety, were cultured in MS medium [21] containing 4.43 g of MS medium, 30 g of sucrose (sugar), 0.1 g of myo-inositol, and 8 g of agar per litre. The pH of the medium was measured and adjusted to 5.7 when necessary. The medium was dissolved in the

microwave oven for 8 min before dispensing 10 ml into test tubes. The test tubes containing the medium were then sterilized in an autoclave. The medium was left to solidify and also left for 3 days in order to ensure lack of contaminants. Seeds were surfaced sterilized by immersing in 1% sodium hypochlorite for 3 sec and were rinsed in distilled water then placed into the growth medium inside the laminar air flow chamber and incubated at room temperature till germination of in-vitro seedling.

Pieces of leaves and stems of in-vitro germinated seedlings were cultured in modified MS medium [24] containing 4.43g of MS medium, 30g of Sucrose, 0.1g of Myo-inositol, 0.1mg of 2, 4- dichlorophenoxy acetic acid, 0.5mg of Kinetin and 8g of Agar per litre with pH adjusted to 5.7 then autoclaved as previously described. The medium was dispensed in to Petri-plates inside the laminar flow hood. The explants were aseptically placed in the medium and incubated at room temperature till visible callus formation.

After callus tissue had developed, each callus was inoculated with 50 individual of *S. bradys*, using a sterile syringe. Plates were maintained at room temperature in the laboratory for 60 days after which the nematodes were extracted for population estimation, using the extraction tray method. The experiment was arranged in a complete randomised design and replicated five times.

#### ***In-vitro* yam plantlet method**

Solid and liquid media were used for this experiment. Cotton wool was used as a support for the yam roots in the liquid media. The liquid media contained 4.43 g Murashige and Skoog basal medium, 100 g Myo-inositol 100, 30 g of Sugar, 1 g Kinetin, 20 g l-cysteine per litre while the solid media contained in addition 2.4 g of Gelrite

In vitro yam plantlets were collected from the genetic laboratory of Crop Protection and Environmental Biology, these were sub-cultured into the prepared solid and liquid media. The medium pH was measured and adjusted to 5.7 when necessary and 10 mls distributed into the autoclavable plastic containers (4 cm base diameter and 5 cm height), these were autoclaved at 121<sup>0</sup>C, for 15 min. the preparation was transferred to the lamina flow hood where the sub culturing was done. The plants were removed from the old culture and placed in the new medium. The culture was left for 5 days to allow for acclimatization. Sterilized nematodes were inoculated into the media very close to the plant root using a sterile syringe.

After inoculation, the *in-vitro* yam plantlets, in both the liquid and solid media were left for 60 days, to allow for two reproductive cycles of the nematode. Plants were thereafter removed from the medium and nematodes were extracted from the yam roots and the medium using the extraction tray method following Coyne *et al* [19]. After extraction, the nematode suspension was concentrated to 50 ml, from which nematodes were assessed as previously described. Following this total number of nematodes and RF were computed as previously described.

### **Yam plants in pot method**

The yam tuber were cut into 50 g setts and planted in saw dust filled into basket, plants were watered every 2 days until setts sprouted. The sprouted yam plants were removed and transplanted into 5 kg pots filled with sterilized soil, this was replicated five times. At two weeks after transplanting, the soil was inoculated with 500 *S. bradys* very close to the plant.

The yam was harvested at 90 days after inoculation when the tubers were taken to the nematology laboratory for extraction of yam nematode. Nematode extraction was carried out on the roots, tuber peels and soil taken from the yam rhizosphere. *Scutellonema bradys* were extracted using the extraction tray method. Reproductive factor (R) was calculated using the formula  $RF = Pf/Pi$ , where Pf is the final nematode population and Pi is the initial nematode population.

## **RESULTS**

### **Effectiveness of culture media in culturing *Scutellonema bradys***

*Scutellonema bradys* were not recovered from yam disc method inoculated on the same day of its preparation and the yam disc inoculated five days after preparation. Population of *S. bradys* was significantly higher in *In-vitro* yam plantlets in solid media than that of yam plantlet in liquid media as presented in Table 1

The reproductive factor of *Scutellonema bradys*, at eight weeks after inoculation reached a value of 2, in two of the culturing methods evaluated in this study. Yam setts planted in pot had a low reproductive factor of 0.56 at the time of evaluation. There was no significant difference between the reproductive factor of *Scutellonema bradys* cultured in *In-vitro* yam plantlet (solid media) and whole yam tuber as shown in Figure 1. The use of kenaf callus as a culturing method for *S. bradys* attained a reproductive factor of 0.8. The yam disc immersed

in agar gave a reproductive factor of 0.46 and a final population of 23, a value lower than the initial population

**Table 1: Population of *Scutellonema bradys* extracted from plant material and media using different culturing methods**

| Culturing method    | Final population |
|---------------------|------------------|
| Tissue culture(SM)  | 103.00±5.61a     |
| Tissue culture (LM) | 71.00±5.10 b     |
| Kenaf callus        | 40.00±2.24 c     |
| Yam disc (SD)       | 0.00± 0.00 e     |
| Yam disc (5D)       | 0.00± 0.00e      |
| Yam disc (agar)     | 23.00±4.06 d     |
| Whole tuber         | 105.00±2.80 a    |
| Yam sett (pot)      | 28±5.98d         |

Means±standard error followed by the same letter are not significantly different at  $p=0.05$  using Duncans's multiple range test; no of replicate =5

SM = Solid media; LM = Liquid media; SD = Yam disc; 5D = yam disc inoculated 5days after preparation; Agar = yam disc placed in agar

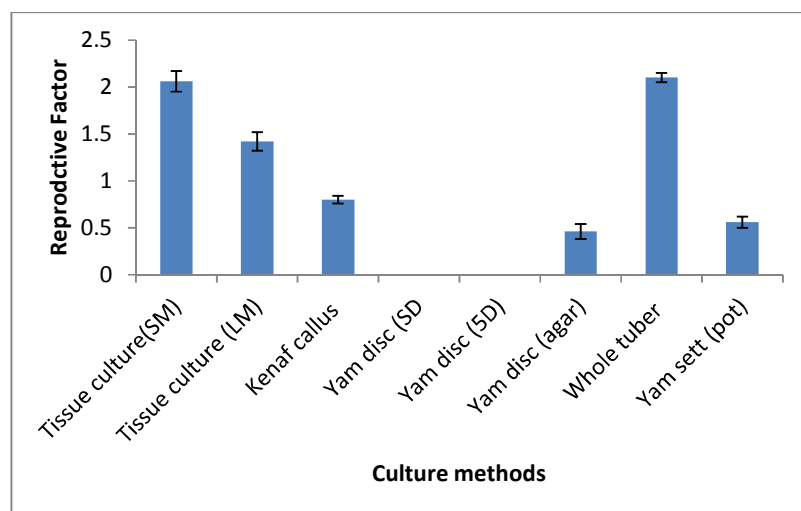


**Fig 1: Reproductive factor of *Scutellonema bradys* in various culturing media**

Values are means of five replicates; bar = standard error; Reproductive factor =  $Pf/Pi$  where  $Pf$  is final nematode population and  $Pi$  is initial population (applied inoculum); SM = Solid media; LM = Liquid media; SD = Yam disc; 5D = yam disc inoculated 5days after preparation; Agar = yam disc placed in agar

229 **DISCUSSION**

230 The study showed that *S. bradys* could survive and reproduce in most of the media used in  
 231 this investigation. The nematodes did not survive in the entire yam disc that were not  
 232 immersed in agar because the yam disc dried out and could not provide a suitable nutrient and  
 233 environment for the nematode survival. This was also observed by Kwoseh [17] where the  
 234 yam slice used rotted. The nematode population doubled at eight weeks after inoculation in  
 235 the yam sett in pot method and the yam tissue culture (solid media). Baimey [22] indicated  
 236 that a reproductive value of 1 means that *Scutellonema bradys* increase in two fold. Part of  
 237 the initial population may have been lost during sterilization or change in environmental  
 238 condition [17]. The surviving sterilised population will then further start new generation. This  
 239 may have been the reason for the low population of *S. bradys* at the end of the experiment.  
 240 Especially given the shorter duration of the experiment compared to Kwoseh [17]. When  
 241 kenaf callus was used for culturing *S. bradys* it was observed that *S. bradys* could multiply in  
 242 the callus as *S. bradys* was recovered from the medium after two months. Yam callus was not  
 243 part of the methods evaluated as protocols because developing yam callus have not been  
 244 reported as dependable for *S. bradys* and this is supported by Baimey [22], who reported  
 245 culturing *S. bradys* on yam callus was unsuccessful as yam leaves induced failed to produce  
 246 callus.

247 The yam disc method where discs were placed directly in petri dishes did not support *S.*  
 248 *bradys* survival. *S. bradys* was not recovered from the yam disc inoculated on the same day  
 249 of disc preparation nor at inoculation five days after yam disc preparation. The five days  
 250 interval between disc preparation and inoculation was to cure the slice and determine if it  
 251 provide a better environment and substrate for *S. bradys*. The yam disc culturing method did  
 252 not support *S. bradys* multiplication as the yam disc dried out. This was also observed by  
 253 Kwoseh [17] when he reported high level of loss of tuber slices which affected their potential  
 254 use as a multiplication medium. However when the yam discs were placed on agar, *S. bradys*  
 255 was extracted at two month after inoculation. The agar appeared to have provided a better  
 256 environment for the nematode activities by providing moisture for the yam disc through the  
 257 culturing period, or that the agar provides additional nutrient for the preservation of the yam  
 258 disc as this was the only yam disc method where the nematode reproduced.

259 The yam sett culturing method gave a RF value less than 1, this indicates that the final  
 260 population is lower than the initial population. Therefore, factors affecting nematode in

locating and penetrating the plant root tissue may be the cause for the low population retrieved [23]. Ways in which the yam disc culturing method can be optimized include peeling in such a way that the preferred peridermal layer in which the nematode usually thrives is not removed with the skin,

Whole yam tuber and *in vitro* yam plantlet methods only, had the RF value of 2 at two months after inoculation, indicating the two options as the best method for culturing *S. bradys* in this study. As effective as tissue culture seems to be in culturing *S. bradys*, the protocol cost is very high. The use of whole tuber for culturing *S. bradys* is a cheaper method as it is efficient, cheap and space conserving. Obtaining Thousands of *S. bradys* from the whole tuber method is possible as Kwoseh [17] obtained 240 folds of *S. bradys* population after five month of storage. This can be done if more cores are made for inoculation on the tubers. However, when tubers availability is a limiting factor, the *in-vitro* yam plantlet method can be explored.

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