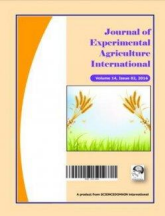

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Evaluation of Culturing Methods for the Yam Nematode *Scutellonema bradys*

Abdulmujib Yusuf[1] and Abiodun O. Claudius-Cole[1*]

[1]Department of Crop Protection and Environmental Biology, University of Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author AY executed the protocol, performed the statistical analyses and wrote the first draft. Authors AOCC designed the study, wrote the protocols, managed the analyses of the study, managed the literature searches and submitted the final draft. Both authors read and approved the final manuscript.

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ABSTRACT

The study evaluated eight culturing methods for *Scutellonema bradys*. The media evaluated for culturing the *S. bradys* were: *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 fitted into a completely randomized design with five replicates and inoculated with fifty individuals of *S. bradys* except for the control. After eight weeks of incubation, *S. bradys* were extracted from the media and plant tissue collected from each method. Final population numbers were estimated and nematode reproductive factor (RF) was calculated. The highest RF value was recorded for the whole yam tuber method and tissue culture method with no significant differences ($p = 0.05$) between the two. *S. bradys* did not multiply in the yam disc methods evaluated. The findings from the study show that the whole yam tuber method was the best option for culturing *S. bradys* while the use of tissue culture plantlets may hold some promise.

Keywords: Callus; culturing; inoculum; population; reproductive factor; tissue culture.

**Corresponding author: E-mail: bi_cole@yahoo.com, b.claudiuscole@gmail.com;*

1. 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 varieties are believed to have originated in the tropical areas of South East Asia, South America and Africa [2]. Yam is the second most important root and tuber crop in Africa and contributes more than 200 calories daily for 60 million people, particularly in the yam belt [3]. 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 [4]. Yam is becoming increasingly important as the major source of saprogenic precursors of cortisone [4]. It is composed mainly of starch, with a small amount of proteins, lipids [5], all the vitamin C required by consumers [6], and the crop is rich in minerals [7].

Yam production is adversely affected by several factors, which include limited availability and cost of planting material, the high cost of labour for operations such as land preparation, staking, weeding, harvesting, storage, pests and diseases [8]. Yam is prone to infection by fungi, viruses and nematodes from the seedling stage through to harvesting and even in storage [9]. It is reported that post-harvest losses of tubers could be as high as 80% [10] and 25% of this could be due to diseases. To a large extent, parasitic attacks directly reflect the biocoenosis of the soil, in that it often includes a complex of bacteria, fungi, nematodes, and insects [11].

Among the nematodes associated with yam cultivation, three are considered to be major constraints namely root-knot nematodes (*Meloidogyne* spp.), the lesion nematode (*Pratylenchus coffeae*) and the yam nematode (*Scutellonema bradys*) [12]. The yam nematode is found in the rhizosphere soil around yam but mainly occurs as a migratory endoparasite of roots and tubers [13]. In the tuber, the nematode is primarily confined to the sub-dermal, peridermal and underlying parenchymatous tissues in the outer 1 to 2 cm of tubers [14] where it feeds intracellularly, rupturing of cell walls and forming cavities [10]. The nematode continues to feed and reproduce in yam tubers stored after harvest [15,16] causing severe dry rot damage [17]. The females lay eggs singly within the tubers and eggs hatch and emerging juveniles feed and go through three subsequent moults to emerge as adults [18]. The duration of the life cycle is about 26 days in a good host [19]. Several reproduction cycles in the tuber can yield up to 6000 nematodes per gram of tuber [20].

Yam is grown mainly as a subsistence crop in Africa [2]. In this system, farmers have largely relied on natural variation for their selection of suitable varieties of yam to cope with the damage caused by plant-parasitic nematode rather than the use of pesticides. Nematode resistant yam cultivars can be one of the most useful, economical and effective means of managing nematodes for resource-poor farmers. The process of identifying resistance involves screening of existing and developing germplasm. Hence the need for a screening protocol that is highly efficient, time-saving and economical. When screening for resistance, or undertaking routine studies with a nematode pest, an important prerequisite is a readily available and consistent supply of the nematode inoculum. Currently, no suitable artificial media or culturing mechanism has been identified for the culturing and production of *S. bradys*. Though nematode species can be artificially cultured on various media such as carrot discs, callus tissue, and tomato roots [21,22], the only documented method that has provided successful multiplication of *S. bradys* was found by Kwoseh [23], who recorded an increase of 240 fold five months after inoculating yam tubers with *S. bradys*.

Infected tubers are not always available for the whole year and can also be difficult to maintain for nematode multiplication. Moreover, yam tuber deterioration due to invasion by fungi, bacteria and mites after nematode infection reduces nematode populations on the tubers [10]. It would, therefore, be useful to have methods that provide a continuous supply of nematodes for experiments during those periods of the year when tubers infected with *S. bradys* will not be available and alternatively provide a pure source of inoculum throughout the year. It was the objective of this study, therefore, to evaluate various culturing methods for *S. bradys* on yam: Using *in vitro* yam plantlet (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 sett planted in potted soil.

2. MATERIALS AND METHODS

2.1 Source of Yam Tubers

Healthy yam tubers of *Dioscorea rotundata* cv *Amula* were collected from the yam barn of the International Institute of Tropical Agriculture (IITA) at Ibadan, Nigeria. Selected yam tubers were seed yam size of 12-15 cm length and 4-6 cm diameter. The tubers used were selected as uniformly as possible. Yam tubers showing symptoms of dry rot, and flaking off of tuber skin characteristic of *S. bradys* infection, were also obtained from IITA Ibadan and a major yam market in Ibadan, Nigeria.

2.2 Extraction of *Scutellonema bradys*

Nematodes were extracted from infected yam tubers showing symptoms of dry rot disease, using the extraction tray method, according to Coyne et al. [24]. The part of the yam showing symptoms of dry rot was peeled, chopped into 3 - 5 mm pieces and placed in the extraction set-up. The chopped diseased yam pieces were then evenly distributed on the tissue. Water was added to the set-up through the area between the sieve and the plate. The set up was left for 72 hours, with daily collection of extracted nematodes and addition of more water. The extracts were all poured into a large 2-litre beaker, then later concentrated by sieving (38µm). The concentrated nematodes were rinsed out into a 250 ml capacity beaker.

2.3 Estimation of Nematode Population in Inoculum

In order to get 50 *S. bradys* per 1 ml, 10 ml of the extract was taken out at each time and 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.

2.4 Sterilization

The glassware were sterilized in an 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 surfaces were wiped with 70% alcohol. Soil was sterilized at 90°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 petri-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.

2.5 Evaluation of Culturing Methods

2.5.1 Yam disc method

Yam tubers were washed thoroughly with soap and rinsed thoroughly with distilled water, then surfaced sterilized 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 lightly, and cut into 0.5 cm thickness, with a diameter of approximately 4 cm, then placed in Petri dishes under the lamina flow chamber. The knife was dipped in ethanol and flamed before each contact. Each yam disc was surface sterilized by dipping it in 10% sodium hypochlorite, then in 70% alcohol and flaming slightly. The yam discs were thereafter placed into sterile glass Petri dishes (9 cm diameter) [25].

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 *S. bradys* immediately after preparation as described by Kwoseh [23]. For the second yam disc method, the yam discs were allowed a period of incubation of five days before inoculation following the procedure for carrot disc culturing method [22]. In the third method, yam discs were placed on agar and then inoculated [26].

Fifty 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 from the whole disc for population estimation using the extraction tray method. After extraction, the nematode suspension was concentrated to 50 ml, and the *S. 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 value was used to estimate the total nematode population extracted per yam disc. Reproductive factor (RF) was calculated using the formula RF = Pf/Pi, where Pf is the final population and Pi is the initial population. This was computed for each of the experiments.

2.5.2 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 dela Cruz et al. [25] as previously described. A sterile cork borer was used to bore three holes of about 10 mm depth and 5 mm diameter into the tuber and the discs were placed in a sterile petri dish and later 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 and labeled. Tubers were placed in a clear plastic container maintained at room temperature in the laboratory for two months. The experiment was arranged in a completely randomised design and replicated five times. The inoculated yam tubers were kept at room temperature (27±2°C) 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, the total number of nematodes and RF were computed as previously described.

2.5.3 Kenaf callus as a medium for culturing nematode

Seeds of kenaf, NHC 15 cultivar were cultured in modified MS medium [27] containing 4.43 g MS medium, 30 g sucrose (sugar), 0.1 g myo-inositol, and 8 g agar per litre. The pH of the medium was measured and adjusted to 5.7 when necessary. The medium was heated in a microwave oven for 8 min allowing the constituents to dissolve 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 them in 1% sodium hypochlorite for 3 sec, rinsed in distilled water, then placed into the growth medium inside the laminar air flow chamber and incubated at room temperature till germination.

Pieces of leaves and stems of the *in vitro* germinated seedlings were cultured in modified medium [27] containing 4.43 g MS medium, 30 g sucrose, 0.1 g myo-inositol, 0.1 mg 2, 4-dichlorophenoxy acetic acid, 0.5 mg kinetin and 8 g agar per litre with pH adjusted to 5.7 then autoclaved as previously described. The medium was dispensed into 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. The experiment was arranged in a completely randomized design and replicated five times. 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 and estimated as previously described.

2.5.4 *In vitro* yam plantlet method (tissue culture)

Solid and liquid media were used for this experiment with yam plantlets. 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 sugar, 1 g kinetin, 20 g l-cysteine per litre while the solid media contained in addition 2.4 g gelrite.

In vitro yam plantlets of *D. rotundata* cv *Amula* were collected from the genetic laboratory of Crop Protection and Environmental Biology, Ibadan and sub-cultured in the prepared solid and liquid media. The pH was measured and adjusted to 5.7 when necessary and 10 ml distributed into the autoclavable plastic containers (4 cm base diameter and 5 cm

height). These were autoclaved at 121°C for 15 min and the preparation transferred to the lamina flow hood where the sub-culturing was carried out. 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. [24]. After extraction, the nematode suspension was concentrated to 50 ml, from which nematodes were assessed as previously described.

2.5.5 Yam plants in pot method

The yam tubers were cut into 50 g setts and planted basket filled with sawdust; plants were watered every 2 days until setts sprouted. The sprouted yam plants were removed and transplanted in 5 kg pots filled with sterilized soil; this was replicated five times. At two weeks after transplanting, 500 *S. bradys* in 1 ml water suspension were applied with a syringe into the soil via two holes at 2 cm depth made at 4 cm from 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 using the extraction tray method. Reproductive factor (RF) was calculated using the formula RF = Pf/Pi, where Pf is the final nematode population (summed from nematodes extracted in all media) and Pi is the initial nematode population.

2.6 Data Analysis

Data collected on nematode counts in root, tubers media or soil were summed per treatment and replicate to give the final nematode population. The data was submitted for analysis of variance and means were separated using the New Duncans multiple range test at p=.05. using the SAS 9.2 software version. Standard error was used to differentiate among the methods for nematode reproductive factor.

3. RESULTS

3.1 Effectiveness of Culture Media in Culturing *Scutellonema bradys*

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

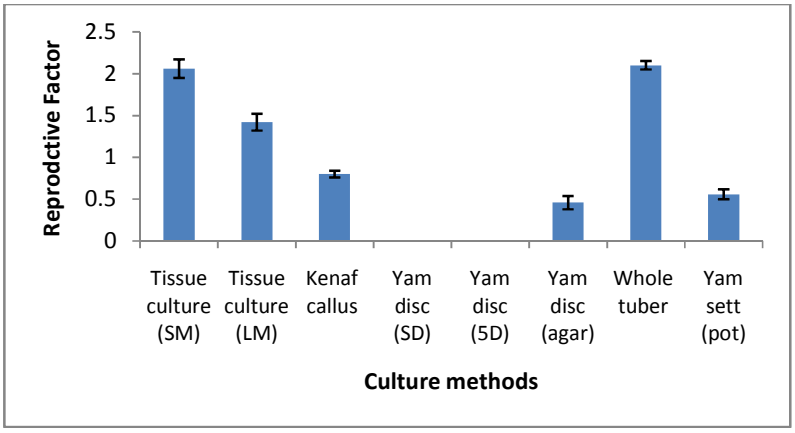

Fig. 1. Reproductive factor of *Scutellonema bradys* in various culturing media

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

The reproductive factor of *S. bradys*, at eight weeks after inoculation, was 2.1 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 *S. bradys* cultured in *in vitro* yam plantlet (solid media) and whole yam tuber as shown in Fig. 1. Kenaf callus as a culturing method for *S. bradys* produced 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 densities
Tissue culture (SM)	103.00±5.61[a]
Tissue culture (LM)	71.00±5.10[b]
Kenaf callus	40.00±2.24[c]
Yam disc (SD)	0.00± 0.0[e]
Yam disc (5D)	0.00± 0.00[e]
Yam disc (agar)	23.00±4.06[d]
Whole tuber	105.00±2.80[a]
Yam sett (pot)	28±5.98[d]

Means±standard error followed by the same letter are not significantly different at p=.05 using Duncan's multiple range test; no of replicates =5
SM = Solid media; LM = Liquid media; SD = Same day inoculation; 5D = inoculation 5 days after preparation; Agar = yam disc placed in agar

4. DISCUSSION

The study showed that *S. bradys* could survive and reproduce in most of the media used in this investigation. The nematodes did not survive in any of the yam discs that were not immersed in agar because the yam discs dried out and could not provide a suitable nutrient and environment for the nematode survival. This was also observed by Kwoseh [23] where the yam slice used rotted. The nematode population doubled at eight weeks after inoculation in the yam sett in pot method and the yam tissue culture (solid media). Baimey [28] indicated that a reproductive value of 1 means that *S. bradys* increase two fold. Part of the initial population may have been lost during sterilization or change in environmental conditions. The surviving sterilized population would require additional time to stabilize before further starting a new generation. This may have been the reason for the low population of *S. bradys* at the end of the experiment, especially given the shorter duration of the experiment compared to Kwoseh [23]. When kenaf callus was used for culturing *S. bradys* it was observed that *S. bradys* could multiply in the callus as *S. bradys* was recovered from the medium after two months. Yam callus was not part of the methods evaluated as Baimey [28] reported that culturing *S. bradys* on yam callus was unsuccessful.

The yam disc method, where discs were placed directly in Petri dishes, did not support *S. bradys* survival. Nematodes were not recovered from the yam disc inoculated on the same day of disc preparation nor from inoculation five days after yam disc preparation. The five days interval between disc preparation and inoculation was to cure the slice and determine if it would provide a better environment and substrate for *S. bradys*. The yam disc culturing method did not support *S. bradys* multiplication as the yam disc dried out. This was also observed by Kwoseh [23] who reported a high level of loss of tuber slices which affected their potential use as a multiplication medium. However, when the yam discs were placed on agar, *S. bradys* was extracted at two months after inoculation. The agar appeared to have provided a better environment for the nematode activities by providing moisture for the yam disc through the culturing period, or because the agar provided additional nutrients for the preservation of the yam disc. This was the only yam disc method where the nematode reproduced. 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 nematodes usually thrive is not removed with the skin the tuber skin could also be left without peeling if an effective sterilization procedure is conducted. *S. bradys* is reported to be confined to the first 2 cm tissue beneath the yam skin [12,29].

The yam sett culturing method gave an RF value of less than 1, indicating that the final population is lower than the initial population. Factors affecting nematodes in locating and penetrating the plant root tissue may be the cause for the low population retrieved [28]. Also, high populations are usually observed during storage and since tubers were not stored, the multiplication cycles were reduced. Only whole yam tuber and one *in vitro* yam plantlet method allowed for reproduction of *S. bradys* by two months after inoculation, indicating the two options as the bests method for culturing *S. bradys* in this study. Although tissue culture seems to be effective in

culturing *S. bradys*, the protocol cost is very high. The use of the whole tuber for culturing *S. bradys* is a preferred method as it is efficient, cheap and space conserving. Obtaining thousands of *S. bradys* from the whole tuber method is possible as Kwoseh [23] obtained a 240 fold increase of the *S. bradys* population after five months of storage. This can be achieved if more cores are made for inoculation on the tubers and with additional storage time. However, when tuber availability is a limiting factor, the *in vitro* yam plantlet method can be explored.

5. CONCLUSION

Among the methods used culturing on whole yam tubers and *in vitro* yam plantlet on semi-solid media were the most effective. Ways of improving or optimizing the yam disc or callus tissue method can improve the nematode yield from them. These methods can be used to produce sufficient quantities of pure cultures of *S. bradys* for experimental purposes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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