

EVALUATION OF CULTURING METHODS FOR the nematode *Scutellonema bradys* IN YAMS

ABSTRACT

The study evaluated eight culturing methods for *Scutellonema 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 the media, plant roots and tubers collected from each method. Final population numbers were was estimated and a reproductive factor (RF) was calculated. The highest RF value was recorded for the in whole yam tuber method and tissue culture method with no significance differences ($p = 0.05$) between the two. *S. bradys* did not survive in the yam disc method evaluated. The findings from the study show that the whole the yam tuber method as is the best option for culturing *S. bradys* while the use of tissue culture plantlets may hold some promise.

Key words: Callus, Inoculum, population, *Scutellonema bradys*, tissue culture, culturing methods

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].

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

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

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

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

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76 **Materials and Methods**

77 **Source of yam tubers**

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

81 **Extraction of *Scutellonema bradys***

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

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90 **Estimation of nematode population in inoculum**

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

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

95 **Sterilization**

96 | The glass-ware were sterilized in an the oven at 160°C for 2 hours. Scalpels and cock borers
97 were autoclaved at 121°C for 15 min before use, and were immersed into 70% alcohol then
98 flamed between use. The working surface was wiped with 70% alcohol. Soil was sterilised at
99 120°C for two hours in the soil sterilizing chamber of the Department of Crop Protection and
100 Environmental Biology, University of Ibadan

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

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109 **Evaluation of culturing methods**

110 **Yam disc method**

111 Yam tubers were washed thoroughly with soap and rinsed thoroughly with distilled water,
112 then surfaced sterilised by rinsing in 1% sodium hypochlorite. A sterile knife was used to cut
113 1-2 cm off both ends of the yam tuber. Tubers were then peeled, and cut into 2 cm diameter
114 | discs into petri dishes under the lamina flow chamber. The knife was dipped in side ethanol
115 and flamed before each contact. Each yam disc was surface sterilized by dipping it in 10%
116 | sodium hypochlorite, then dipping in 70% alcohol and flaming slightly. The yam discs were
117 thereafter placed into sterile petri dishes [20].

118 The yam disc method procedure was varied to give three different yam disc methods. In the
119 | first yam disc method, the yam discs were inoculated with *S. cutellonema bradys*
120 immediately after preparation. For the second yam disc method, the yam discs were allowed a
121 period of incubation of five days before inoculation. In the third method, yam discs were
122 | placed in agar and then inoculated.

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

133 Whole yam tuber method

134 Yam tubers of different cultivars (?) were washed thoroughly with soap and rinsed properly
135 with distilled water, then surfaced sterilized by rinsing in 1% sodium hypochlorite. The
136 nematode inoculum was sterilized using the method of Delacruz *et al.* [20] as previously
137 described. A sterile cork borer was used to bore three holes of about 10 mm depth and 5 mm
138 diameter into the tuber and the discs were placed in a sterile petri dish and later, this was
139 used to cover the hole after inoculation with nematodes.

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

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

151 Kenaf callus as a medium for culturing nematode

152 Seeds of kenaf, NHC 15 cultivar variety, were cultured in MS medium [21] containing 4.43
153 g of MS medium, 30 g of sucrose (sugar), 0.1 g of myo-inositol, and 8 g of agar per litre. The

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154 pH of the medium was measured and adjusted to 5.7 when necessary. The medium was
155 heated in a microwave oven for 8 min allowing the constituents to dissolve **was dissolved in**
156 **the microwave oven for 8 min** before dispensing 10 ml into test tubes. The test tubes
157 containing the medium were then sterilized in an autoclave. The medium was left to solidify
158 and also left for 3 days in order to ensure lack of contaminants. Seeds were surfaced sterilized
159 by immersing them in 1% sodium hypochlorite for 3 sec **and were** rinsed in distilled water,
160 then placed into the growth medium inside the laminar air flow chamber and incubated at
161 room temperature till germination. **of in-vitro seedling.**

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

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

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173 **In-vitro yam plantlet method (tissue culture)**

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174 Solid and liquid media were used for this experiment with yam plantlets. Cotton wool was
175 used as a support for the yam roots in the liquid media. The liquid media contained 4.43 g
176 Murashige and Skoog [21] basal medium, 100 g **M**myo-inositol 100, 30 g **of** Sugar, 1 g
177 **K**kinetin, 20 g l-cysteine per litre while the solid media contained in addition 2.4 g **of**
178 **g**Gelrite

179 In vitro yam plantlets were collected from the genetic laboratory of Crop Protection and
180 Environmental Biology, Ibadan and **these were** sub-cultured **into** the prepared solid and
181 liquid media. The medium pH was measured and adjusted to 5.7 when necessary and 10 **mls**
182 distributed into the autoclavable plastic containers (4 cm base diameter and 5 cm height),
183 **t**These were autoclaved at 121⁰C, for 15 min. **and** the preparation **was** transferred to the
184 lamina flow hood where the sub- culturing was done. The plants were removed from the old

185 culture and placed in the new medium. The culture was left for 5 days to allow for
186 acclimatization. Sterilized nematodes were inoculated into the media very close to the plant
187 root using a sterile syringe.

188 After inoculation, the in-vitro yam plantlets, in both the liquid and solid media, were left for
189 60 days, to allow for two reproductive cycles of the nematode. Plants were thereafter
190 removed from the medium and nematodes were extracted from the yam roots and the medium
191 using the extraction tray method following Coyne *et al.* [19]. After extraction, the nematode
192 suspension was concentrated to 50 ml, from which nematodes were assessed as previously
193 described. Following this total number of nematodes and RF were computed as
194 previously described.

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195 Yam plants in pot method

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

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

207 RESULTS

208 Effectiveness of culture media in culturing *Scutellonema bradys*

209 *Scutellonema bradys* specimens were not recovered from yam disc methods iwhere they were
210 inoculated on the same day of its preparation and the yam disc inoculated five days after
211 preparation. The Ppopulation of *S. bradys* was significantly higher in in vitro In-vitro yam
212 plantlets in solid media than that of on yam plantlets in liquid media as presented in Table 1.

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213 The reproductive factor of *S. cutellonema bradys*, at eight weeks after inoculation, reached a
214 value of 2, in two of the culturing methods evaluated in this study. Yam setts planted in pot

215 had a low reproductive factor of 0.56 at the time of evaluation. There was no significant
 216 difference between the reproductive factor of *S. cutellonema* *bradys* cultured in *In-vitro* yam
 217 plantlet (solid media) and whole yam tuber as shown in Figure 1. The use of kenaf callus as a
 218 culturing method for *S. bradys* attained a reproductive factor of 0.8. The yam disc immersed
 219 in agar gave a reproductive factor of 0.46 and a final population of 23, a value lower than the
 220 initial population.

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

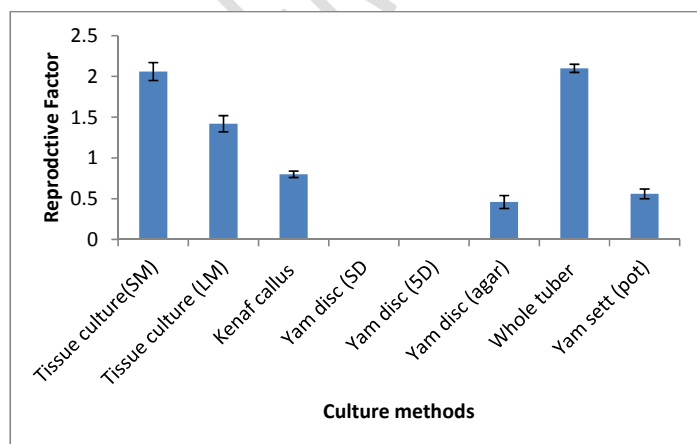
Culturing method	Final population
Tissue culture (SM)	103.00±5.61a
Tissue culture (LM)	71.00±5.10 b
Kenaf callus	40.00±2.24 c
Yam disc (SD)	0.00± 0.00 e
Yam disc (5D)	0.00± 0.00e
Yam disc (agar)	23.00±4.06 d
Whole tuber	105.00±2.80 a
Yam sett (pot)	28±5.98d

223 Means±standard error followed by the same letter are not significantly different at $p=0.05$
 224 using Duncan's multiple range test; no of replicates =5
 225

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

Comment [GJ<8]: How can SD = Yam disc when you have Yam disc (SD) in the table?

Comment [GJ<9]: In the Table you need a space before SM and a bracket after SD Also "Culture methods" is not in the middle



229
 230
 231 **Fig 1: Reproductive factor of *Scutellonema bradys* in various culturing media**

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

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237 DISCUSSION

238 The study showed that *S. bradys* could survive and reproduce in most of the media used in
239 this investigation. The nematodes did not survive in the entire yam disc that **were was** not
240 immersed in agar because the yam disc dried out and could not provide a suitable nutrient and
241 environment for the nematode survival. This was also observed by Kwoseh [17] where the
242 yam slice used rotted. The nematode population doubled at eight weeks after inoculation in
243 the yam sett in pot method and the yam tissue culture (solid media). Baimey [22] indicated
244 that a reproductive value of 1 means that *S. cutellonema bradys* increase **in** two fold. Part of
245 the initial population may have been lost during sterilization or change in environmental
246 conditions [17]. The surviving sterilised population will then further start **a** new generation.
247 This may have been the reason for the low population of *S. bradys* at the end of the
248 experiment. **E**specially given the shorter duration of the experiment compared to Kwoseh
249 [17]. When kenaf callus was used for culturing *S. bradys* it was observed that *S. bradys* could
250 multiply in the callus as *S. bradys* was recovered from the medium after two months. Yam
251 callus was not part of the methods evaluated as **protocols because developing yam callus**
252 **have not been reported as dependable for *S. bradys* and this is supported by** Baimey
253 [22], **who** reported **that** culturing *S. bradys* on yam callus was unsuccessful, **as yam leaves**
254 **induced failed to produce callus.**

255 The yam disc method, where discs were placed directly in petri dishes, did not support *S.*
256 *bradys* survival. **It *S. bradys*** was not recovered from the yam disc inoculated on the same day
257 of disc preparation nor **at from** inoculation five days after yam disc preparation. The five
258 days interval between disc preparation and inoculation was to cure the slice and determine if
259 it provide a better environment and substrate for *S. bradys*. The yam disc culturing method
260 did not support *S. bradys* multiplication as the yam disc dried out. This was also observed by
261 Kwoseh [17] **who when he** reported **a** high level of loss of tuber slices which affected their
262 potential use as a multiplication medium. However, when the yam discs were placed on agar,
263 *S. bradys* was extracted at two months after inoculation. The agar appeared to have provided
264 a better environment for the nematode activities by providing moisture for the yam disc

265 | through the culturing period, or because that the agar providesd additional nutrientss for the
266 | preservation of the yam disc, as tThis was the only yam disc method where the nematode
267 | reproduced.

268 | The yam sett culturing method gave a RF value of less than 1, this indicatinges that the final
269 | population is lower than the initial population. Therefore, fFactors affecting nematodess in
270 | locating and penetrating the plant root tissue may be the cause for the low population
271 | retrieved [23]. Ways in which the yam disc culturing method can be optimized include
272 | peeling in such a way that the preferred peridermal layer in which the nematodess usually
273 | thrivess is not removed with the skin,

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

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