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Full Length Research Paper

Observations on *in vitro* behaviour of the zygotic axes of fluted pumpkin

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Fluted pumpkin, *Telfairia occidentalis* Hook. f., is an important leaf and seed vegetable and a local medicinal plant across West Africa. Many biological constraints have become potent threats to the existence of the plant necessitating an urgent need to collect and conserve the existing narrow genetic diversity. However, conservation by seed storage is impossible because the seed is recalcitrant, that is desiccation- and chilling-sensitive. Micropropagation is the only immediate alternative option for the conservation of fluted pumpkin germplasm. In order to facilitate this, the behaviour of excised embryonic axes and shoot tips of fluted pumpkin under *in vitro* conditions were investigated. Systemic infection of seeds from field led to frequent and high microbial contamination in culture. There was interaction between the type of microbial contamination and the storage environment of seeds prior to excision of the axes. Axes greened under low light intensity and root growth was dependent on the orientation of the axes. In general, zygotic axes of the plant are easy to grow *in vitro* under a range of nutrient media and culture conditions.

Key words: Fluted pumpkin, embryonic axes, greening, microbial contamination.

INTRODUCTION

Telfairia occidentalis Hook. f. (fluted pumpkin) is a leaf and seed vegetable that is well-known for its high nutritional, medicinal and economic potentials in the coastal areas of West Africa. The succulent, tasty leaves and stems, and nutritious seeds make it the most popular vegetable to millions of people, ranking as one of the three most widely eaten vegetables at homes and in restaurants across Nigeria (Abiose, 1999). Young shoots and leaves of fluted pumpkin are cooked, alone or in mixtures with other vegetables, and used as soups for different kind of starchy dough. Fresh leaf concoction is a high-value health tonic for the treatment of acute anaemia (Akoroda, 1990a; Schippers, 2000). Immature seeds, eaten cooked or roasted, are preferred to mature ones because anti-nutrients increase with maturity (Akw-aowo et al., 2000). Seed cotyledons are also processed into seasonings, high-protein cake, marmalade, infant weaning foods, flour bread supplement and different local fermented foods (Egbekun et al., 1998; Giami and Isichei 1999; Steinkraus 2002; Giami et al., 2003). Seeds are believed to have lactation-promoting properties and are in high demand by nursing mothers (Schippers, 2000). Mature seeds are a good source of edible unsaturated oil (Esuoso et al., 2000, Giami et al., 1999; Nkang et al., 2003). Roots have high alkaloid content and their extracts are therefore used for controlling pest and rodents (Akubue, 1980; Ajibesin et al., 2002). Therefore, the plant is the major income earner for many subsistence families and features prominently in trans-border trade especially among Nigeria, Cameroon and Benin Republic.

Non-availability of seed for planting is a major constraint to the growing and widespread interest in the mono-cropping and large scale production for the economic parts (Odiaka and Schippers, 2004). Fluted pumpkin can be propagated only by seeds, but seeds germinate and rot inside the fruits even before harvesting and they cannot be stored for long periods because they

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are sensitive to both desiccation and chilling (Ajayi, 2005; Ajayi et al., 2006). Although these constraints had been noted long ago (Esiaba, 1982), they have persisted because scant attention had been paid to propagation and conservation problems, compared with the utilization of fluted pumpkin (Longe et al., 1983; Giami and Bekebain 1992; Egbekun et al., 1998; Esuoso et al., 2000). This is largely due to the lack of expertise and information on handling the unequivocally recalcitrant seeds of fluted pumpkin (Odiaka and Schippers, 2004; Ajayi et al., 2006).

Recalcitrant seeds are desiccation sensitive and therefore shed in a hydrated condition. Hence, they cannot be dried for storage nor frozen without lethal damage. They die at high axis water concentration when dehydrated slowly, although axes withstand more water loss on rapid dehydration (Pammenter et al., 1998). Recalcitrant seeds of individual species may also be damaged by low temperatures, this is chilling-sensitivity. These seeds are usually stored in hydrated condition for short- to medium-term duration while cryostorage appears to be the only option for the long-term (Berjak and Pammenter, 2004). Hence, much of the work on genetic resources conservation of recalcitrant-seeded species utilizes embryonic axes, which are capable of regenerating whole plants in culture. Therefore, plant *in vitro* technology offers a potential solution for the long term storage of germplasm of plant species with recalcitrant seed behaviour. The failed attempt to root vine cuttings *in vitro* (Balogun et al., 2002) was aimed at developing a protocol for vegetative propagation in order circumvent this problem. A search of the literature did not yield any information on the *in vitro* culture of fluted pumpkin. In view of the potential of tissue culture for both long-term germplasm conservation and genetic improvement of fluted pumpkin, observations on a series of experiments designed to understand *in vitro* behaviour of the excised axes of fluted pumpkin are hereby reported.

MATERIALS AND METHODS

Seed materials

Treatments and storage conditions for seeds prior to *in vitro* culture have been described in detail by Ajayi et al. (2006). Zygotic axes used in this study were carefully excised to prevent injury.

Culture medium

Two media were prepared by varying concentrations of nutrients: full- and half-strength MS nutrients (Murashige and Skoog, 1962) each with 0.8% agar and 3% sucrose. Five axes were cultured on a Petri dish to form a replication and each treatment was replicated four times. The cultures were kept under a 16 h/8 h light/dark photoperiod at 23-25°C at two light intensities, 34 and 67 ± 9.8 µE m^{-2} s^{-1}. Full-strength MS was eventually used in all subsequent investigations.

Sterilization

A two-step surface sterilization with CaOCl at different combinations and durations were first tried in order to asses the effectiveness of the concentrations as surface disinfectant and tolerance of fluted pumpkin axes to the treatments. Axes were initially excised with a large cotyledonary attachment for the first step and then trimmed down to the final size for the second step. The combinations were i) sterile distilled water- control, ii) 1%-2 min, 0.5%-1 min, iii) 1%-5 min 0.5%-2.5 min, iv) 2.5%-2 min 1.25%-1 min, v) 2.5%-5 min 1.25%-2.5 min. The two-step axis trimming was reduced to one and a higher concentration tried as follows vi) 5%-5min, vii) 5%-10 min, viii) 10%-5 min, and ix) 10%-10 min. The good tolerance of all these CaOCl treatments by the axes and the ineffectiveness of these treatments to curtail both fungal and bacterial contamination necessitated another sterilization protocol. In a new three-step protocol, excised axes were treated with bactericide- 1% Hibitane (a.i. Chlorhexidine gluconate, Zeneca South Africa) followed by a systemic fungicide solution comprising 0.05% Early Impact (a.i. flutriafol and carbendazim, Zeneca Agrochemicals, South Africa) and 0.25% Previcur (a.i. propamocarb-hydrochloride, R.T. Chemicals, South Africa). Axes were treated in succession with both for 10 min and finally for 5 min with 2.5% CaOCl, to which two drops of a wetting agent had been added. When the isolation of fungal inoculum from the culture of internal cotyledon segments suggested systemic infection of the axes and the degree of microbial contamination after the above sterilization protocol further increased with storage, the three step sterilization was modified by increasing the concentrations (5 and 10%) and duration (5 and 10 min) of sterilization with CaOCl. The systemic fungicide solution described above and kanamycin (Sigma-Aldrich, St. Louis, USA) at 50 µ ml^{-1} were incorporated into the culture medium. The fungicide solution was co-autoclaved with the medium, while antibiotics were thereafter added through sterile, bacteria-proof filters (Millex®GP, Carrigtwahill, Co Cork, Ireland), pore size 0.22 µm to the medium after cooling. These combinations were tested on a cohorts of axes from freshly harvested fruits and seeds and which were cultured on medium into which fungicides had been incorporated.

Fungi isolation

Mycelia of proliferating fungi were isolated from the seed coat using colour and other characteristics to distinguish the species. They were grown on potato dextrose agar in plates kept in the dark a 25±3°C and sub-cultured repeatedly until a pure culture of each type was obtained. The pure cultures were sent to the Mycology Unit, Biosystematics Division of the ARC- Plant Protection Research Institute, Pretoria, South Africa for professional identification.

Micropropagation

Three concentrations of each of two growth hormones, NAA at 0.1, 0.5 and 1.0 mg l^{-1} and kinetin at 1.0, 2.0 and 5 mg l^{-1}, were combined in a factorial design (Table 1) and added to full- strength MS medium, 0.8% agar and 3% sucrose in order to find a suitable medium for *in vitro* shoot tip and axillary bud cultures. Root tips of excised zygotic axes were trimmed off and cotyledon segments were trimmed down as much as was possible before culture. Similarly, axillary buds were excised from the three topmost leaf axils of elongating stems. The shoot tips and axillary buds were surfaced sterilized using the three-step protocol described above.

Table 1. Tolerance of mature fluted pumpkin zygotic axes to and effectiveness of different sterilization protocol.

	Control Sterile dH_2O	1%-2 min 0.5%-1 min	1%-5 min 0.5%-2.5 min	2.5%-2 min 1.25%-1 min	2.5%-5 min 1.25%-2.5 min	5%-5min	5%-10 min	10%-5 min	10%-10 min	3-step	3-step + Antibiotics
After two weeks storage at 16°C											
Days to germination	4a	4a	4a	4a	4a	4a	4a	7b	7b	5c	22d
% germination	100	100	100	100	100	100	100	100	100	100	100
% infection	40a	10b	0c	10b	0c	10b	10b	10b	0c	0c	0c
After four weeks storage at 16°C											
Days to germination	5a	5a	5a	5a	5a	8bc	9b	9b	9b	7c	25d
% germination	100	100	100	100	100	100	100	100	100	100	100
% infection	100a	85b	80b	85b	80b	80b	80b	80b	80b	75b	50c

Figures on the same row with same letter are not significantly different at $P<0.05$.

Figure 1. Half-cotyledon of fluted pumpkin showing the relative size of embryonic axis.

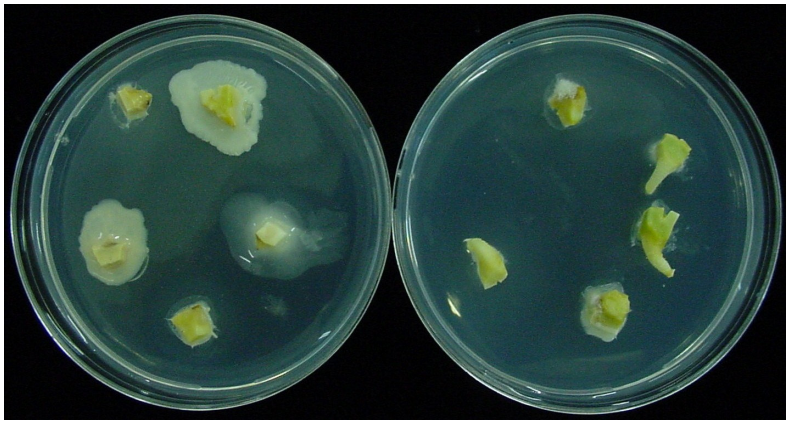


Figure 2. Influence of storage temperature on type of contamination in culture of excised axes.

RESULTS

The zygotic axis of fluted pumpkin was very small compared with seed size. In freshly extracted seeds, axes excised with as minimal cotyledonary segment as possible constituted on average 0.3% and 0.2% of seed fresh and dry mass, respectively (Figure 1). Similarly, cotyledons and seed coat constituted 83 and 16.7% of fresh mass and, 80 and 19.8% of dry mass, respectively. There was no visible physical separation between axes and cotyledons in freshly harvested and hydrated seeds and the bluntly pointed root end was not always a good guide in trimming the cotyledons to avoid injury to the axes. However, cotyledons shrank as seeds lost moisture leaving a tiny visible hole on the shoot tip at the fusion of cotyledons with the axes.

The zygotic axes of fluted pumpkin were able to tolerate different combinations of CaOCl concentrations and duration up to 5% for 10 min. Although 10% did not appear to have had a significantly different effect on viability compared with 5%, it prolonged time to germination. Growth of axes extracted from freshly harvested seeds was very fast and rate of growth progressively slowed down with increasing duration of storage. Microbial contamination increased with duration of seed storage prior to excision of axes. By four weeks after storage, degree of contamination of axes was about 80%. Incorporation of fungicides and antibiotics into the nutrient media did not eliminate infection but reduced the level to 50% and delayed the time to first manifestation of infection by at least one week. Generally, axes excised from seeds stored at 6°C showed more bacterial than fungal contamination while fungal proliferation in culture predominated in those from seeds stored at 16°C (Figure 2). The dominant fungi isolated from the seeds were *Clonostachys roseus* and *Fusarium solani*.

Germinating axes greened only under low light intensity and the intensity of greenness was higher for partially-desiccated, compared with non-desiccated axes (Figure 3). Despite its effect on the type of microbial contamination in culture, temperature in which seeds had been stored prior to excision and in vitro culture did not affect greening of germinating axes. Growth of whole axes on media fortified with fungicides and antibiotics was considerably slowed down- development and elongation after six weeks (Figure 4a) was comparatively less than growth after two weeks on media without fortification (Figure 4b). Generally, root growth in culture, compared with shoot growth, was either abnormal or retarded when whole embryonic axes or shoot tips were cultured perpendicular to the culture surface (Figure 3). However, this abnormality was not observed in axes and

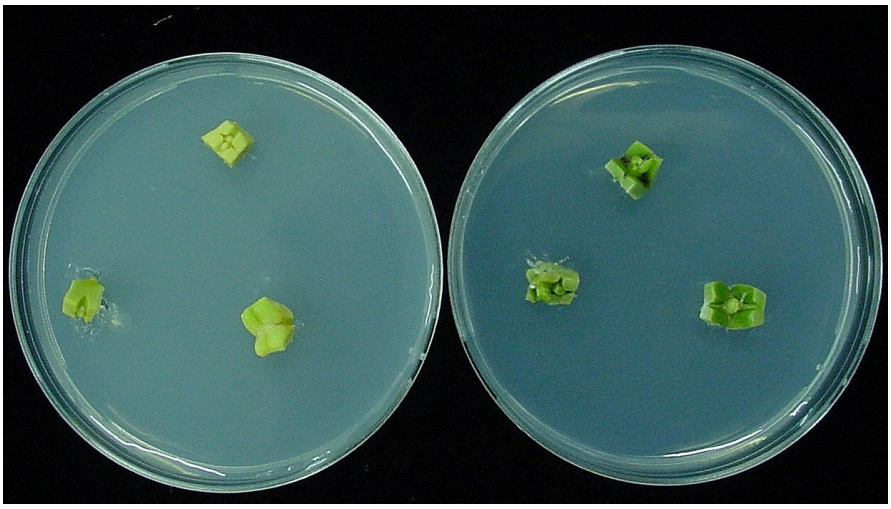


Figure 3. Greening of *in-vitro* grown non-desiccated (1.83 g g^{-1}) versus fast-desiccated axes (0.71 g g^{-1}).

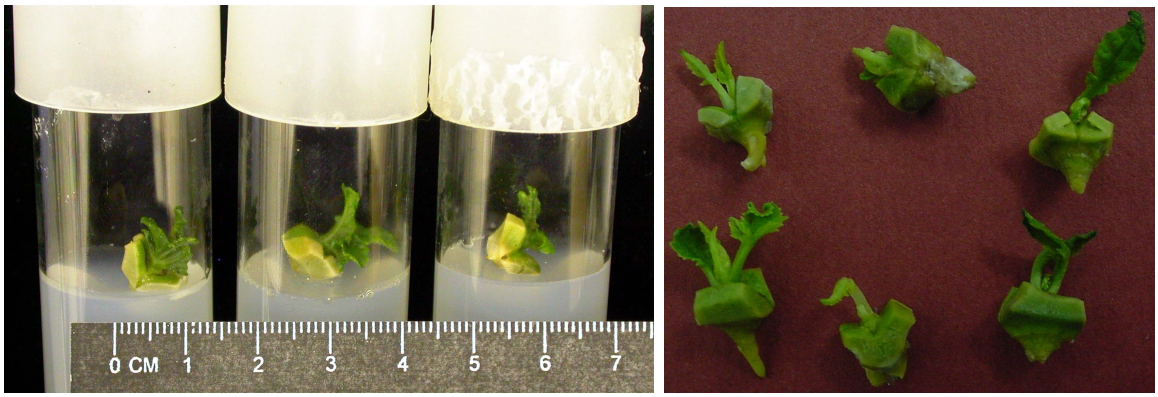


Figure 4. Zygotic axes (a) 42 days after culture on a fungicides + antibiotic-fortified medium and (b) 13 days axes after culture on a media without fortification.

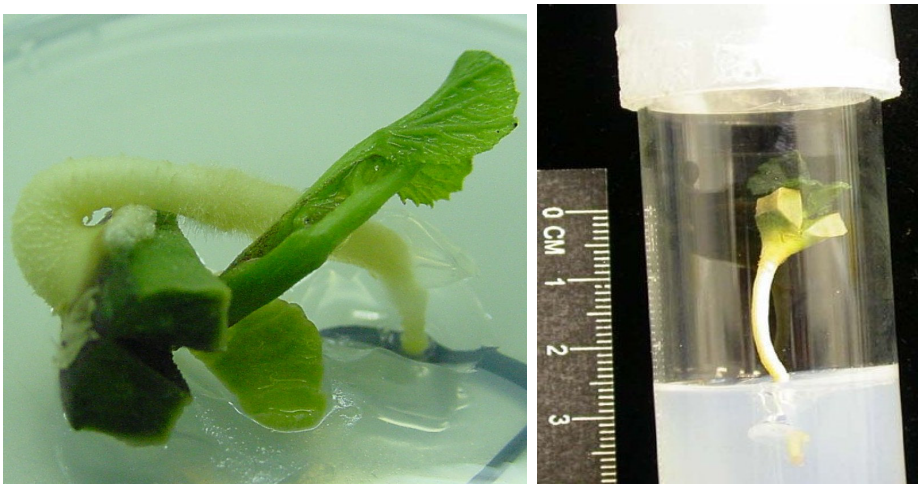
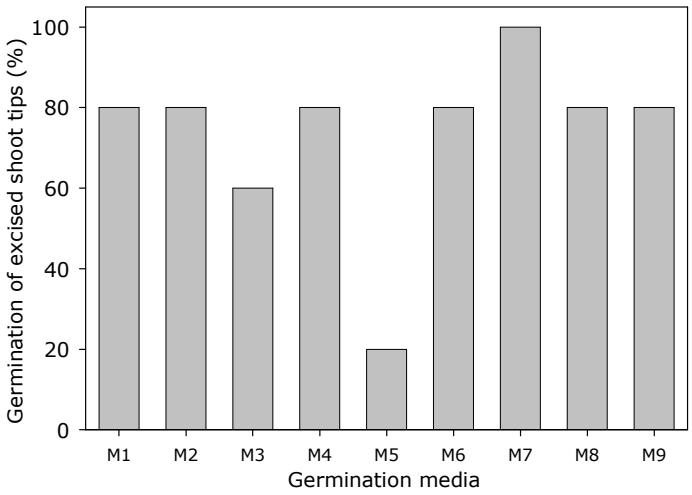


Figure 5. Normal root growth after changing orientation of axes on culture media fortified with fungicides and antibiotic (a) and without (b).



Media Combinations	NAA (mg l^{-1})	Kinetin (mg l^{-1})
M1	0.1	1.0
M2	0.1	2.0
M3	0.1	5.0
M4	0.5	1.0
M5	0.5	2.0
M6	0.5	5.0
M7	1.0	1.0
M8	1.0	2.0
M9	1.0	5.0

Figure 6. Germination of embryonic shoot tips on different media combinations

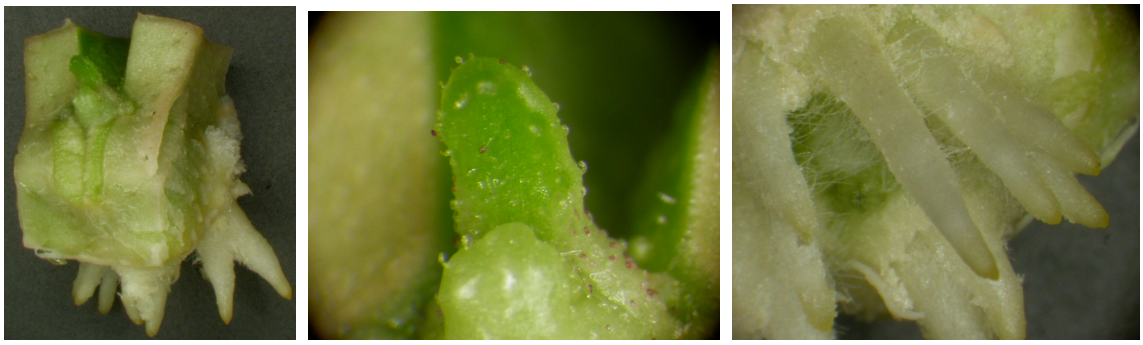


Figure 7. *In vitro* growth of excised shoot tips grown on MS medium fortified with 0.1 mg l^{-1} NAA and 2.0 mg l^{-1} kinetin (a), germinating shoot end (b) and rootlets radially growing on the periphery of the axes at the root end (c).

tips cultured horizontally and in abnormal plantlets it was corrected by changing the orientation of the root end (Figure 5). Compared with plantlets with abnormal root development, growth of plantlets with simultaneous and proportional growth of both root and shoot was accelerated.

In vitro growth of excised axillary buds was not stimulated on any of the nine media formulations tested. Rather, all the explants callused. Similarly, the axillary buds did not respond to the medium prepared for bud-break. 80-100% initiation of germinative growth of excised embryonic shoot tips was observed on seven out of the nine media combinations, while 60 and 20% shoot growth initiation were observed on two other media (Figure 6). The characteristic greening was also observed on tips that initiated growth (Figure 7). But root growth, to different degrees, was observed on only five out of the seven media combinations. The best normal growth was observed on full strength MS medium supplemented with 0.1 mg l^{-1} NAA and 2.0 mg l^{-1} kinetin (M2).

DISCUSSION

Tissue culture offers prospects for the long-term conservation of fluted pumpkin germplasm given the recalcitrant and non-storable post-shedding behaviour of the seeds (Ajayi et al., 2006). Excised zygotic axes of fluted pumpkin responded well to *in vitro* conditions. Excised full axes and embryonic shoot tips from freshly extracted seeds grew easily and rapidly on full strength MS medium supplemented with 0.8% agar and 3% sucrose. However, the rapid *in vitro* growth of the axes of freshly extracted seeds poses a problem for maintaining the cultures for any considerable length of time. On the other hand, delayed extraction of seeds from fruits and excision of axes into culture reduces vigour of the plantlets and also predisposes the culture to a higher level of microbial contamination. Therefore, techniques of reduced-growth storage of germplasm that combines reduction of nutrient in the media with reduced incubation temperature and modification of gaseous environment

(Ng and Ng, 1991) could be employed to overcome this problem. Similarly, the culture of axes on half-strength MS fortified with 0.8% agar and 1.5% sucrose, reduction of cotyledonary attachment to the axes and incorporation of fungicides and antibiotic into the media all slowed down growth with the first two options being less effective than the last. The greening of the axes which suggests photosynthetic capacity deserves further investigation as a potential reduced growth strategy. Although apparently normal, germinated axes had not elongated more than 2 cm after six weeks on a media to which kanamycin and systemic fungicides had been incorporated. Orientation of the axes to prevent root elongation is another *in vitro* behaviour of fluted pumpkin axes that can be utilized to slow down plantlet growth and elongation.

It is a general practice to culture axillary buds with part of the subtending leaf to supply endogenous hormones for growth in culture but this was practically impossible in the case of fluted pumpkin plants grown in the green house and field. The leaf petiole is subtended at about 60° and often longer than 10 cm, with an additional 5-15 cm leaf length depending on stage of development. These problems can be circumvented by using axillary buds from *in vitro* grown seedlings after sufficient elongation of the seedlings in a suitable culture media.

The chilling-sensitivity of the associated fungi, implied by the predominance of bacteria associated with axes of seeds stored at 6°C, and their isolation from internal cotyledon tissues of externally uninfected seeds suggest a high likelihood of systemic infection of seeds prior to storage. Fungi account for up to 95% and bacteria 5% loss of fluted pumpkin fruits during storage under ambient condition (Odiaka and Schippers, 2004). Fungal proliferation from endogenous inoculum has been shown to be promoted during hydrated storage in highly recalcitrant seeds (e.g. Calistru et al., 2000; Berjak et al., 2003). When axes from stored seeds were cultured *in vitro*, the rate of infection was usually relatively low (1-20%) in the first two to four weeks of storage followed by a significant rise, up to 90%, thereafter. Incorporation of systemic fungicides and a broad-spectrum antibiotic (Kanamycin at 50 µg/ml) did not completely eliminate infection but reduced it to about 50%. Even though the fungicides and the antibiotic have been shown to be effective against the principal fungi and bacteria associated with some species of South African provenance (Calistru et al., 2000), a broader range of fungicides and antibiotics needs to be tested against the microflora infecting *T. occidentalis* seeds. Alternatively, fungicides and antibiotics could emerge as an essential factor in the production fluted pumpkin seeds.

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