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Short communication

Beneficial effect of arbuscular mycorrhizas on acclimatization of micropropagated cassava plantlets

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Abstract

Survival and development of cassava (*Manihot esculenta* Crantz) plantlets has been increased by modifying tissue culture protocols and by mycorrhizal inoculation. About 90% of plantlets were successfully rooted in vitro and 75% survived after the acclimatization phase. Inoculation with *Glomus deserticola* early in the post vitro weaning stage enhanced percent survival and improved tolerance to the transplanting stress. Shoot, root and tuber development of the micropropagated plants was increased following inoculation with different arbuscular mycorrhizal (AM) fungi. Growth responses were dependent on both the cultivar (clone) and the AM fungi involved. *G. deserticola* was very effective in improving growth of both clones, the effectivity of *G. clarum* and *G. fasciculatum* being dependent on the cultivar. These results emphasize the need for selection trials before appropriate plant cultivar/AM fungi combinations can be recommended.

Keywords: *Glomus*; *Manihot*; Micropropagation; Mycorrhizas

1. Introduction

Cassava (*Manihot esculenta* Crantz) is one of the most important crops in some developing countries (Roca and Thro, 1993). Its starchy tuberous roots produce more calories per unit of land than any other crop, apart from sugarcane. This highly

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productive source of energy is available all year round by means of sustainable, low input, agricultural practices and under environmental conditions adverse for other crops. Cassava is well adapted to a wide range of tropical and subtropical environmental stress and develops well on very poor soils (Allemann et al., 1993).

Although cassava is easily propagated through traditional agronomic practices, programmes to improve its propagation and to select more appropriate varieties have been set up. A key tool in these programmes is tissue culture and micropropagation of appropriate plant material to enable target clonal selection and mass production of plantlets with remarkable agronomic attributes and improved resilience to environmental stress. In this context, another biotechnological tool that should be considered is the manipulation of mycorrhizas because the establishment of this symbiosis can enhance the tolerance to transplant stress, particularly if the plant material derives from in vitro culture. In fact, arbuscular mycorrhizas have been shown to play a decisive role in the acclimatization of micropropagated plants (Hooker et al., 1994).

Studies on in vitro culture of cassava have been developed with different purposes, such as the obtention of virus-free plants (Kaiser and Teemba, 1979; Delgado and Rojas, 1993), or to induce somatic embryogenesis and plant regeneration (Szabados et al., 1987; Stamp and Henshaw, 1987; Taylor and Henshaw, 1993). Inherent to the micropropagation techniques, there is often a transplant stress during the post vitro weaning stage, with the subsequent death of plantlets. As far as we know, a 55% survival rate is the highest level reported for cassava (Zok, 1993).

Cassava, as many tropical plantation crops do, exhibits a mycotrophic habit and a considerable degree of dependence on this symbiosis for optimal development (Sieverding, 1991). Therefore, together with the improvement of tissue culture protocols, AM inoculation programmes must be developed to improve outplanting survival and performance. Since both the media used for the in vitro stages (obviously sterile) and the substrates for subsequent plantlet growth, either in potting mixes or in fumigated nursery beds, usually lack mycorrhizal propagules, inoculation of in vitro propagated plantlets early during the post vitro weaning stage could improve acclimatization and subsequent growth (Gianinazzi et al., 1990; Lovato et al., 1996; Azcón-Aguilar and Barea, 1997).

The objective of the present study was to develop experimental approaches aimed to produce cassava cultivars (in vitro plantlets) with an optimized mycorrhizal status. In this context, this paper reports on the development of appropriate protocols for tissue culture and on the effects of selected AM fungi inoculation on survival and growth of the micropropagated plant material.

2. Material and methods

2.1. Plant material

Four clones of cassava (*M. esculenta* Crantz) were selected because of their resistance to drought and saline stress. These were: clone SOM-1, originally from Somalia and supplied by the Instituto Italiano de Oltremare and the Instituto per la Propagazione delle Specie Legnose (CNR), both in Florence (Italy); clone 05 (Identifica-

tion number 7902), originally from Ivory Cost (IDESSA) and supplied by ORSTOM, Montpellier (France); clone 35 (Identification number 60444), originally from Nigeria (IITA) and supplied by ORSTOM, Montpellier (France) and clone 50 (Identification Bonova rouge 1), originally from Ivory Cost (IRAT Mouaké) and supplied by ORSTOM, Montpellier (France).

2.2. Micropropagation protocol

Nodal explants (1–1.5 cm long) having one axillary bud and derived from in vitro plant material of the four cassava clones were used. These initial explants from each cassava clone were aseptically transferred to test tubes (25 × 120 mm) containing MS mineral formulation (Murashige and Skoog, 1962) supplemented with sucrose (87.6 mM) and inositol (11.1 mM), but lacking vitamins. The influence of the addition of NAA on rooting was investigated. Consequently, 100 explants from each clone were cultured on the MS mineral formulation added with sucrose and inositol and another 100 explants on the same medium but supplemented with NAA (0.7 μM). All the explants were grown for 45 days to achieve growth and rooting in the same experimental medium.

Growth conditions were 25 ± 1°C, with a 16-h photoperiod (Sylvania Gro-Lux fluorescent lamps) which provided a photosynthetic photon flux (PPF) of 30 μmol s^{-1} m^{-2}.

After 45 days the explants have both sprouted and rooted allowing their transplant to ex vitro conditions for the acclimatization stages. The rooted plantlets were individually transferred from the test tubes to 300 ml pots containing a mixture of moist peat: perlite (3:1, v:v). Plants were grown in a growth chamber at 22°C with a 16-h photoperiod and a PPF of 111 μmol s^{-1} m^{-2} and gradually exposed to reduced relative humidity by progressively removing a plastic cover. After 2 weeks, the plants were placed in a greenhouse where the temperature ranged from 18 to 25°C and the relative humidity from 80 to 60%.

At the end of the in vitro phase, the percentage of rooted explants, number and length of the roots formed, shoot length and number of buds produced were determined for each plantlet. The percentage of survival after the acclimatization phase was also recorded 4 weeks after the plants were transferred to the greenhouse.

2.3. Mycorrhizal inoculation experiments

For the mycorrhizal studies, only plantlets obtained in the media without NAA addition and from clone 50 and SOM-1 were used. Two experiments were carried out as aimed: (I) to study the effect of the AM fungus *Glomus deserticola* on the acclimatization of rooted plantlets and (II) to test the effect of different mycorrhizal fungi (*G. clarum*, *G. deserticola*, *Entrophospora colombiana* and *G. fasciculatum*) on the development of cassava plantlets, already acclimatized.

In both experiments, inocula consisted of washed mycorrhizal onion roots with the external mycelium and spores attached, but free from soil particles. A total of 1 g (fresh weight) of this inoculum, cut into 1 cm fragments, was applied to each plantlet close to

the root system. Filtered (Whatman 1 filter paper) leachates of the root inoculum were applied to the non-inoculated control plantlets to compensate for the free-living microbiota associated with the mycorrhizal inoculum (Vidal et al., 1992).

In Experiment I, mycorrhizal inoculation was performed at the beginning of the acclimatization stage (see Section 2.2 above), when plantlets were transferred from the test tubes to open-pot conditions, using the peat:perlite substrate already described. Mycorrhizal inoculum was placed in the planting hole at transplanting. Inoculated and non-inoculated (control) plants were grown in a growing chamber, as above described and transferred 2 weeks later to greenhouse conditions. Percentage of survival was recorded after a growing period of 8 weeks in the greenhouse. Twenty replicates were prepared for each treatment and clone.

In Experiment II, plants already acclimatized were inoculated when transferring from the growth chamber to the greenhouse. At this stage, plants were transplanted to 1 kg-pots containing a soil:sand (1:1, v:v) mixture. This potting mixture was previously steam-sterilized (100°C for 1 h during 3 consecutive days). The soil consisted of clay, 42.5%; silt, 34.0%; sand, 23.5%; organic matter, 1.18%; total N, 0.14%; and contained extractable P at 6.1 mg kg^{-1} and K at 0.7 mg kg^{-1}. The soil was taken from the experimental field at the Estación Experimental del Zaidín. The plants were inoculated by mixing the inoculum of the corresponding AM fungus with the soil:sand substrate, or were left as non-inoculated controls. Eight replicates per treatment and clone were prepared.

Once in the greenhouse, plants from both experiments were grown for 8 weeks under day/night temperatures ranging from 18 to 25°C. Daylength natural photoperiod was extended to 16 h daily by cool-white fluorescent lamps. Plantlets were fertilized weekly with 10 ml of full-strength Long Aston nutrient solution (Hewitt, 1952) lacking P and watered when necessary.

In Experiment I, only the percentage of plant survival was determined. In Experiment II dry weight of shoots, roots and tubers was recorded after harvesting and drying for 25 h at 65°C. The percentage of mycorrhizal root length was assessed in stained root samples (Phillips and Hayman, 1970), by using a gridline intersect technique (Giovannetti and Mosse, 1980).

Student's *t*-test was used for the statistical analysis of the data. In the case of the values given as percentages, data were subjected to an arcsin square-root transformation to ensure homogeneity of variances.

3. Results

Table 1 summarizes the results obtained in the development of the cassava clones during the micropropagation process. There were no significant differences in shoot growth, number of buds per explant and number of explants rooted in the growth media tested (with or without auxin). The addition of NAA improved the number of roots formed, although these roots were shorter than those in the medium without NAA. The presence of NAA affected negatively the acclimatization rate of clone 35, benefited that of clone 50 and did not affect significantly that of clones SOM-1 or 05. In general

Table 1
Organ development and survival of four micropropagated cassava clones

Parameter	Clone and media							
	SOM-1		05		35		50	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
No. of explants used	100	100	100	100	100	100	100	100
Shoot length (cm)	6.5a	4.9a	6.1a	5.0a	5.6a	5.6a	6.0a	5.9a
No. of buds per explant	3.7a	2.2a	3.5a	3.5a	3.7a	4.2a	3.5a	3.9a
No. of explants rooted	99a	98a	89a	90a	85a	95a	92a	93a
No. of roots per explant	4.6a	6.8c	3.3b	4.7a	3.3b	5.4a	3.2b	5.2a
Mean root length (cm)	3.9a	2.8b	4.4a	3.1b	3.6a	1.6b	3.9a	2.8b
No. of plant acclimatized post vitro	82.0b	70.0b	65.2c	62.2c	97.5a	80.0b	65.2b	82.2b

A = Modified MS basal medium without plant growth regulators.
B = Modified MS basal medium supplemented with NAA (0.7 μM).
Values for each parameter not sharing a letter in common differ significantly ($P \leq 0.01$).

survival of the acclimatized plants in both media was higher than that reported in the literature (Roca, 1984; Zok, 1993; Bhagwat et al., 1996).

Fig. 1 shows the survival rate of cassava plants inoculated with *G. deserticola* at the beginning of the acclimatization stage. Although the percentage of survival of the plantlets from both clones was relatively high for the uninoculated controls, mycorrhizal inoculation improved it significantly. A positive mycorrhizal effect was already evident at 21 days of the acclimatization process (results not shown), for either clone SOM-1 or 50.

Results recorded in Fig. 2 clearly show that *G. clarum*, *G. deserticola* and *G. fasciculatum* improved plant growth after establishing an adequate symbiotic relationship with the micropropagated (and already acclimatized) SOM-1 cassava clone. *G. deserticola* and *G. fasciculatum* were also effective at improving growth of clone 50,

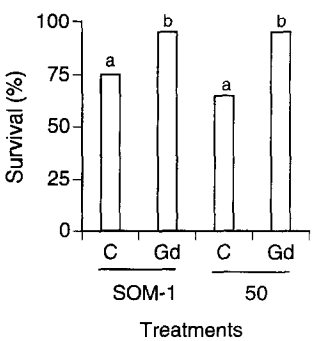

Fig. 1. Effect of mycorrhizal inoculation (C = Control, Gd = *G. deserticola* inoculated) on the survival of two clones ('50' and 'SOM-1') of cassava plants. The mycorrhizal inoculum was applied at transplanting from the test tube, at the beginning of the post vitro stage. Mean values (20 replicates) not sharing a letter in common differ significantly ($P \leq 0.05$).

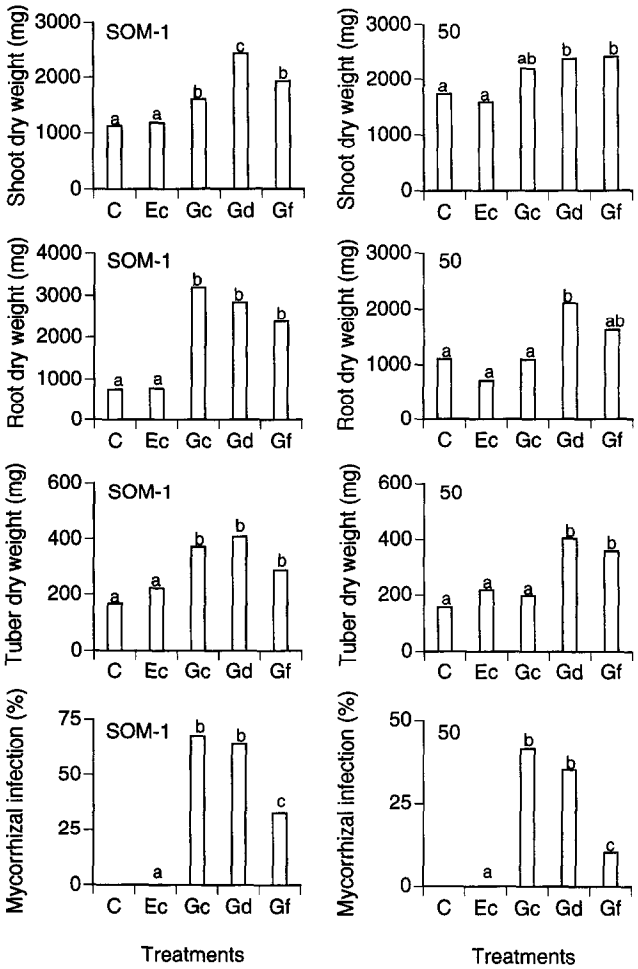

Fig. 2. Effect of different mycorrizal fungi on the growth of two clones ('50' and 'SOM-1') of already acclimatized cassava plants. C = Control, Ec = *E. colombiana*, Gc = *G. clarum*, Gd = *G. deserticola*; Gf = *G. fasciculatum*. Mean values (eight replicates) not sharing a letter in common differ significantly ($P \leq 0.05$).

whereas *G. clarum*, in spite of producing the highest level of root colonization, did not improve significantly plant development. *G. fasciculatum* colonized the root system of both cassava clones in a lesser extent than the other two AM fungi. However, it produced a higher growth response in clone 50 than *G. clarum*.

As indicated above and after a previous selection (data not included), the potting media chosen for the studies on the mycorrhizal effect on plant growth included sterilized soil, a substrate component that usually improves AM formation and effectivity in micropropagated plants (Gianinazzi et al., 1990; Vidal et al., 1992). The apparent beneficial effect of the presence of soil in the growing medium on mycorrhiza formation by plants produced in vitro appears to be confirmed by the present results. *E.*

colombiana, however, did not colonize the roots of any of the clone tested and, consequently, did not affect plant growth (Fig. 2).

4. Discussion

The survival rate and development of micropropagated plantlets from different cassava clones have been improved by introducing AM fungi able to establish effective mycorrhizal symbiosis with the plant.

The addition of NAA to the growing medium did not affect significantly shoot development nor the number of rooted explants for any of the clones tested. However, the number and length of the roots produced in vitro was influenced by the auxin, in the sense that more roots, although shorter, were induced in presence of the hormone. This differential rooting pattern did not have any important consequence for the further acclimatization of the SOM-1 and 05 clone plantlets. For clones 35 and 50 the differences in root development, as a consequence of NAA addition, led to a decreased (clone 35) or increased survival rate (clone 50) after the acclimatization phase. It is therefore difficult to reach a general conclusion about the effect of the auxin on the acclimatization of the cassava plantlets.

For both clones tested the percentage of survival after acclimatization was higher than values reported for other cassava cultivars (Roca, 1984; Zok, 1993; Bhagwat et al., 1996). In addition, mycorrhizal inoculation increased the survival rate, helping the plantlets to resist the environmental stress induced at transplanting from axenic conditions to normal cultivation in open pots. This positive effect of AM fungal inoculation has been shown for other plant/growing condition combinations (Gianinazzi et al., 1990; Vidal et al., 1992; Barea et al., 1993; Hooker et al., 1994).

Mycorrhizal formation with appropriate AM fungi increased significantly the development of the cassava clones. A similar AM effect on plant growth has been described for micropropagated plants of the tropical crop *Annona cherimola* Mill when inoculated with mycorrhizal fungi after the acclimatization phase (Azcón-Aguilar et al., 1994). Because typical arbuscules were formed, AM was probably functioning by its recognized role of improving nutrient acquisition by the host plant.

The effects of mycorrhizal inoculation on plant growth may also be due to improved rhizogenesis, as has been suggested for other crops (Schubert et al., 1990). Since AM fungi produce plant hormones (Barea and Azcón-Aguilar, 1982), they might improve root formation and development of the in vitro-produced cassava plantlets.

Growth responses were dependent on both the cultivar (clone) and the AM fungus involved. *G. deserticola* was very effective in improving growth of both clones, as it was, although in a lower extent, *G. fasciculatum*. The effectivity of *G. clarum* was dependent on the cassava cultivar. Differences in the effect of each species of AM fungi on different host plant cultivars or clones is a typical feature in mycorrhizal studies and particularly for micropropagated plantlets (Varma and Schüepp, 1994). These results emphasize the need for selection trials before appropriate plant cultivar/AM fungi combinations can be recommended.

E. colombiana, an AM fungus usually described as very effective at improving growth of cassava plants (Howeler et al., 1987), did not affect plant performance in the conditions of the present study. In fact, *E. colombiana* failed to colonize the root system of cassava plantlets. A possible explanation for this lack of colonization of *E. colombiana* in already acclimatized cassava roots may be related to the age of the plants at the inoculation time. Differences in the ability to form mycorrhizas related to plant age and soil type, have been commonly described (Hepper, 1985; Barea et al., 1993).

This paper, therefore, contributes to the current idea that the application of the mycorrhizal biotechnology can benefit the production of micropropagated plantlets of target crops (Hooker et al., 1994). Particularly, for cassava cultivars the proposed methodology appears transferable, easy to handle under simple inputs and feasible for the ecophysiological conditions of the tropical area where cassava is a key crop (Roca and Thro, 1993). In such conditions, mycorrhizas would improve the survival, establishment, nutrient uptake and resistance to stress situations to help plant development (Barea and Jeffries, 1995; Howeler et al., 1982).

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