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Original Research Article

In vitro response of three contrasting cassava (M*anihot esculenta* Crantz) varieties to mannitol-induced drought stress

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Abstract

In vitro selection of drought-tolerant cassava varieties is essential for rapid breeding for drought tolerance. The objectives of this study were to determine the response of three contrasting cassava varieties to mannitol-induced drought stress to establish its suitability for *in vitro* screening and examine relationships among growth parameters. Plantlets were raised from nodal segments on Murashige and Skoog (MS) medium containing 0 (control), 5, 10, 15, 20, 25 and 30 g/l mannitol. Variety CH 140 had the highest survival of explants and frequency of root formation, while MV 99/0395 recorded the highest number of chlorotic leaves and the lowest survival of explants. The lowest numbers of leaves were produced at 25 and 30 g/l mannitol by the three varieties. In CH 140, the highest number of leaves was produced in medium free of mannitol, while the highest number of leaves was produced at 5 and 10 g/l mannitol in MV 99/0395 and TMS 01/1206, respectively. In TMS 01/1206, number of roots produced decreased as the concentration of mannitol in culture media increased, whereas in CH140, number of roots increased as the concentration of mannitol increased before decreasing; while in MV 99/0395, number of roots was not affected by an increase in mannitol concentration. As the concentration of mannitol in the culture media increased shoot height of plantlets decreased with a sharp decline at 20 mg/l mannitol. Concentration of mannitol and survival of explants had significant negative correlation with all parameters. However, frequency of root formation only had significant positive correlation with shoot length. The study concluded that differential responses were expressed by the three varieties to mannitol-induced drought stress and mannitol at 20 g/l concentration was a suitable *in vitro* drought inducing-agent for screening cassava varieties for drought tolerance.

Keywords: growth; moisture deficit; relationship; root crop; screening.

INTRODUCTION

Cassava is a major source of carbohydrates for approximately 500 million people in developing regions of the tropical world (Bull et al., 2011). Cassava is consumed in various forms by humans and livestock (Vandegeer et al., 2012). It is used for the production of starch, pharmaceuticals, glucose syrup, agrochemicals and ethanol (Vandegeer et al., 2012; Asante-Pok, 2013). In addition, fresh cassava leaves are used as vegetable and its stems as stakes to promote vine growth and photosynthesis in yam production, and as firewood. There are several reasons for a significant increase in cultivation of cassava (FAO, 2013). First, cassava is a hardy crop because of its ability to thrive on poor soils and withstand stress at 4–6 months after crop establishment (El-Sharkawy, 2004). Second, the crop is suitable for low-external-input cropping systems practiced in most of the developing countries (FAO, 2013). Third, cassava has unique starch properties, such as excellent clarity, high gelatinization potential and bland flour. Starch from cassava is pure, as it contains very low quantities of ash, crude protein and fat (Sanchez et al., 2009). Finally, it provides flexibility in harvesting, as tubers can be stored in the soil for a fairly long period, which facilitates cassava processing and marketing (Nassar and Ortiz, 2010). However, several biotic and abiotic constraints, such as drought, pests, diseases, low soil fertility, shortage of planting materials, post-harvest physiological deterioration and access to

markets, limit cassava production (Asante-Pok, 2013; FAO, 2013).

A major impact of climate change is drought or water deficit, which imposes limited-water environment on plants (Trenberth et al., 2014). Global monitoring and analysis of climatic variables have provided evidence that the world, including countries where cassava is cultivated, is experiencing climate change (IPCC, 2007). Under drought conditions, water available for plant uptake for metabolic reactions falls below requirement, thus adversely impacting growth and physiological processes. The effects of water deficit on cassava plants are many and vary depending on length and intensity of drought and stage of growth of the plant (Aina et al., 2007). Cassava is sensitive to soil water deficit during the first three months of growth after planting (Bergantin et al., 2004; Aina et al., 2007). Water-deficit stress during this period significantly reduces the growth and development of both the primary roots and shoots (El-Sharkawy and Cadavid, 2002). Water stress impacts tuber development, even if the drought stress is alleviated later (Bergantin et al., 2004). This finally results in low tuber yield at the normal maturity time of the crop and delays harvest (El-Sharkawy and Cadavid, 2002; Bergantin et al., 2004). Furthermore, tuber formation is difficult under water-deficit conditions; a study has shown that a 45% reduction in leaf formation resulted in 83% and 97.8% reduction in tuber yield and starch content, respectively (Vandegeer et al., 2012). Similarly, nitrogen–use efficiency of cassava experiencing drought is decreased as a result of the partitioning of higher proportion of nitrogen to root biomass than to shoot biomass (Aina et al., 2007). Vandegeer et al. (2012) reported that cyanide content increased three-fold in young leaves and four-fold in tubers of water-stressed cassava (Vandegeer et al., 2012). Therefore, identification of water-deficit stress-tolerant varieties for use in breeding programs is desirable.

Field selection of cassava lines for drought tolerance in a conventional breeding program is a long and time-consuming exercise (Turyagyenda et al., 2013). This situation is compounded by heterozygosity, clonal propagation and poor flowering in most of the elite cassava varieties and landraces (Ceballos et al., 2004). Therefore, there is a need to use a faster than conventional breeding approach, such as *in vitro* and molecular techniques, to identify cassava varieties tolerant to water-deficit stress (Turyagyenda et al., 2013). *In vitro* technique allows screening of a large number of varieties in relatively small space within a short period. In addition, the method is not affected by season or environmental conditions. Mannitol is an effective osmotic agent suitable for *in vitro* screening of crop for tolerance to water-deficit stress (Lipavská and Vreugdenhil, 1996; Abdel-Raheem et al., 2007; Hassanein, 2012). Mannitol is a six carbon sugar alcohol synthesized by plants and other organisms. It has been found to be effective as a water stress-inducing agent in soybean (Neto et al., 2004), garlic (Ikeda et al., 2002), *Pelargonium* spp. (Hassanein, 2012), and tomato (Abdel-Raheem et al., 2007). However, there is no information on the use of mannitol as drought stress-inducing agent in cassava. The hypothesis tested was that the three cassava varieties would express differential responses to *in-vitro* mannitol-induced drought stress and thus establish mannitol's suitability for *in vitro* screening of cassava germplasm for drought tolerance trait. The objectives of this study were to (i) determine morphological responses of three contrasting cassava varieties to mannitol-induced drought stress *in vitro* (ii) determine the best concentration of mannitol to screen cassava varieties for drought stress and (ii) examine relationships among growth parameters.

MATERIALS AND METHODS

Plant materials and growth conditions

Plantlets of three cassava varieties contrasting for drought tolerance, viz., CH140 (drought tolerant), TMS01/1206 (intermediate) and MV99/0395 (drought sensitive) were obtained from the *in vitro* germplasm collection of Tissue Culture Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan. The plantlets were maintained on MS medium (Sigma-Aldrich, St. Louis, MO; Murashige and Skoog, 1962) supplemented with 30 g/l sucrose, 7 g/l agar (Sigma-Aldrich, St. Louis, MO), 100 mg/l myo-inositol, 0.05 mg/l benzylaminopurine and 0.01 mg/l naphthalene acetic acid. The medium was adjusted to pH 5.7 before autoclaving for 20 min at 121 ^{0}C. The culture conditions were 26 ± 1 °C, 16-h photoperiod, and 25 µmol·m^{-2}·s^{-1} irradiation provided by Philips 32-W cool white fluorescent lamps (Philips Electric Company, Hyderabad, India). Subculturing was done at 4-week intervals.

In vitro response of cassava to mannitol-induced drought stress

Nodal stem segments obtained from three-week-old *in vitro* plantlets of the three varieties were cultured on MS medium supplemented with 30 g/l sucrose, 7 g/l agar, 100 mg/L myo-inositol, 0.05 mg/L benzine aminopurine, 0.01 mg/L naphthalene acetic acid and 0, 5, 10, 15, 20, 25 and 30 g/l concentration of mannitol. The 3 × 7 factorial experiment was arranged in a completely randomized design with three replicates. Fifteen explants were used per treatment. Cultures were kept in a growth chamber at 25 ± 2 °C and 16-hour photoperiod (irradiation = 90 µmol m^{-2} s^{-1}). Survival of explants (SURV), number of green leaves (NGL), number of chlorotic leaves (NCL), frequency of root

Table 1. Mean squares of parameters obtained from *in vitro* response of cassava to mannitol-induced water deficit stress

SV†	DF†	SURV†	NCL†	NGL†	SL†	RF†	NRP†	ARL†	NSE†
Replicate	9	18.5	0.58	20.66	35.52	21.6	4.75	13.67	0.29
Variety(V)	2	23.6**	6.02**	36.49**	2.76 ^{NS}	28.4**	13.61**	31.46**	0.19 ^{NS}
Mannitol(M)	6	19.4**	1.25 $^{NS\,s}$	2.05**	2.35**	15.8**	0.56**	1.80**	0.17 ^{NS}
V X M	12	8.34 ^{NS}	2.36 ^{NS}	7.46**	1.45 ^{NS}	3.46 ^{NS}	2.34**	6.35**	0.37*
Error	126	2.24	1.14	2.04	1.81	2.21	0.55	1.29	0.17
R^2 (%)		62	24	48	44	42	50	53	22

* = Significant at P = 0.05 probability level, ** =Significant at P = 0.01 probability level, NS = not significant, †SV = Source of variation, DF = Degrees of freedom, SURV = Survival of explants, NCL = Number of chlorotic leaves, NGL = Number of green leaves, TNL = Total number of leaves, SL = Shoot length, RF = frequency of root formation, NRP = Number of root per plantlet, ARL = Average root length per plantlet, NSE = Number of shoot per explant.

Table 2. Main effect of variety on explants survival, number of chlorotic leaves and root formation of three cassava varieties

Variety	SURV† (%)	NCL†	RF† (%)
TMS01/1206	66.7 ± 0.8b	1.5 ± 0.1a	24.3 ± 2.1b
CH 140	88.0 ± 0.9a	1.4 ± 0.1b	51.3 ± 2.8a
MV 99/0395	65.5 ± 0.5b	1.5 ± 0.1a	24.3 ± 1.9b
Mean	73.4	1.5	33.3

†Values are means (± standard error) of three replicates. Means followed by different letters in same column are significantly different at 5% probability level according to Tukey Test. †SURV = Survival of explant (%), RF = Root formation (%), NCL = Number of chlorotic leaves.

formation (RF), number of roots per plantlet (NRP), average root length per plantlet (ARL), number of shoots per explant (NSE) and shoot length (SL) were recorded after five weeks of culture initiation.

Statistical analysis

Data were subjected to analysis of variance using PROC GLM of the Statistical Analysis Systems (SAS Institute 2002). Means were separated by Tukey's test at 5% level of probability. Simple linear correlation and regression analyses were used to show relationships among growth parameters.

RESULTS AND DISCUSSION

In vitro response of cassava to mannitol-induced drought stress

Mean squares from two-way analysis of variance showed that interaction of cassava variety and concentration of mannitol was significant ($P < 0.01$) on the number of green leaves, number of roots, average root length and number of shoots (Table 1). This implies that the values of those parameters in each variety depend on concentration of the mannitol because the variety responded differently to each concentration of mannitol. Thus, decision on selection of cultivar should not be based solely on main effects of cultivar or concentration of mannitol when these parameters are considered; rather interaction means should be examined. Furthermore, the main effect of cassava variety significantly ($P < 0.05$) influenced survival of nodal segment (explants) in culture medium, number of chlorotic leaves and frequency of root formation. Similarly, the main effect of concentration of mannitol was significant on shoot length, survival of nodal segment and frequency of root formation (Table 1). Significant main effects of cultivar and concentration of mannitol observed in this study could be attributable to ability of cassava cultivars to mitigate oxidative stress differently according to severity of moisture stress, which manifested in observed variations in responses. Similar influence of cultivar, duration and severity of moisture stress and stage of growth on physiological, morphological and molecular responses has previously been reported in field and greenhouse screening for drought tolerance in cassava (Okogbenin et al., 2013; Turyagyenda et al., 2013) and in *in vitro* screening of other crops, such as soybean, garlic, *Pelargonium* spp. and tomato (Ikeda et al., 2002; Neto et al., 2004; Abdel-Raheem et al., 2007; Hassanein, 2012).

Among the varieties, CH 140 had the best *in vitro* growth performance in terms of survival of explants and frequency of root formation by explants, while MV 99/0395 recorded the highest number of chlorotic leaves and the lowest survival of explants (Table 2). The outstanding performance of CH 140 in terms of explant survival, number of green leaves, shoot length, frequency of root formation and number of root per plantlet as a high drought tolerant variety is expected. The same is true for TMS 01/1206 and MV 99/0395 as intermediate and low drought tolerant varieties. The differences in water stress tolerance induced by mannitol exhibited by the three cassava varieties in the present study could be related to differences

Table 3. Main effect of mannitol concentration on explants survival, shoot height and root formation of three cassava varieties

Concentration of mannitol (g/l)	SURV† (%)	RF† (%)	SL (cm) †
0	100.0 ± 0.0a	86.7 ± 4.1a	3.7 ± 0.3a
5	92.0 ± 4.5b	60.3 ± 5.3b	2.7 ± 0.3b
10	88.3 ± 3.8c	46.7 ± 4.5c	1.8 ± 0.1c
15	78.3 ± 3.6d	26.7 ± 3.6d	1.3 ± 0.1d
20	70.7 ± 4.2e	6.3 ± 0.5e	1.0 ± 0.1e
25	60.3 ± 4.4f	3.3 ± 0.3e	0.6 ± 0.1f
30	48.3 ± 3.6g	3.3 ± 0.3e	0.3 ± 0.1g

†Values are means (± standard error) of three replicates. Means followed by different letters in same column are significantly different at 5% probability level according to Tukey Test. SURV = Survival of explants, RF = Root formation, SL = Shoot length.

Table 4. Interaction of variety and concentration of mannitol on some growth parameters of in vitro plants

		NGL†	NRP†	ARL† (cm)	NSE†
TMS 01/1206	0	4.50 ± 0.67c	0.10 ± 0.10e	0.13 ± 0.13g	1.00 ± 0.00c
	5	4.70 ± 0.52c	0.70 ± 0.30b	0.73 ± 0.32d	1.40 ± 0.16a
	10	5.30 ± 0.42b	0.50 ± 0.27c	0.96 ± 0.50c	1.30 ± 0.21b
	15	4.20 ± 0.47c	0.00 ± 0.00f	0.00 ± 0.00h	1.40 ± 0.16a
	20	2.30 ± 0.40e	0.00 ± 0.00f	0.00 ± 0.00h	1.10 ± 0.10c
	25	1.31 ± 0.12f	0.00 ± 0.00f	0.00 ± 0.00h	1.00 ± 0.00c
	30	0.63 ± 0.11g	0.00 ± 0.00f	0.00 ± 0.00h	1.00 ± 0.00c
CH 140	0	6.31 ± 0.83a	0.20 ± 0.13d	0.27 ± 0.18f	1.10 ± 0.10c
	5	4.00 ± 0.26c	2.00 ± 0.45a	3.71 ± 0.83a	1.00 ± 0.00c
	10	3.60 ± 0.31d	2.10 ± 0.48a	2.77 ± 0.67b	1.00 ± 0.00c
	15	4.20 ± 0.42c	0.60 ± 0.31b	0.46 ± 0.20e	1.20 ± 0.13b
	20	4.30 ± 0.58c	0.40 ± 0.27c	0.73 ± 0.55d	1.30 ± 0.21b
	25	3.00 ± 0.31d	0.15 ± 0.05d	0.51 ± 0.01e	1.02 ± 0.20c
	30	2.05 ± 0.15e	0.15 ± 0.05d	0.33 ± 0.02f	1.02 ± 0.20c
MV 99/0395	0	4.10 ± 0.62c	0.10 ± 0.00e	0.13 ± 0.13g	1.00 ± 0.00c
	5	4.40 ± 0.58c	0.10 ± 0.10e	0.30 ± 0.30f	1.50 ± 0.22a
	10	3.50 ± 0.58b	0.00 ± 0.00f	0.00 ± 0.00h	1.30 ± 0.15b
	15	2.40 ± 0.22e	0.10 ± 0.10e	0.05 ± 0.05h	1.00 ± 0.00c
	20	1.40 ± 0.37f	0.10 ± 0.10e	0.04 ± 0.04h	1.00 ± 0.00c
	25	1.01 ± 0.01f	0.10 ± 0.10e	0.00 ± 0.00h	1.00 ± 0.00c
	30	0.65 ± 0.02g	0.10 ± 0.10e	0.00 ± 0.00h	1.00 ± 0.00c

†Values are means (± standard error) of three replicates. Means followed by different letters in same column are significantly different at 5% probability level according to Tukey Test. †NGL = Number of green leaves, NRP = Number of root per plantlet, ARL = Average root length per plantlet, NSE = Number of shoot per explant.

in their ability to detoxify reactive oxygen species. According to Nayyar and Gupta (2006) the capacity to degrade reactive oxygen species differs among plant species and between varieties of same species, which explains differences in their stress tolerance. As the concentration of mannitol in the culture media increased, survival of explants, frequency of root formation by explants and shoot height of plantlets decreased with a sharp decline at 20 m/l mannitol (Table 3). Plant growth and gene expression in response to water stress are highly dose-dependent, suggesting the existence of very sensitive machinery assessing the stress level and fine-tuning molecular, physiological and biochemical responses (Chaves et al., 2003). Above 20 g/l mannitol, it is possible that plantlet ability to synthesis osmotically active compounds could had stopped leading to loss of plantlets, chlorophyll degradation and growth cessation. Thus, 20 g/l mannitol is the best concentration for *in vitro* screening of cassava as explant survival is key to *in vitro* screening.

The number of green leaves, number of roots, average root length and number of shoots of the three cassava varieties responded differently to concentrations of mannitol (Table 4). The lowest numbers of leaves were produced at 25 and 30 g/l mannitol in the three varieties; whereas in CH 140, the highest number of

Table 5. Coefficients of correlation among growth parameters and regression coefficients (b) from regression of growth parameters on mannitol concentration

Parameter	CONMA	SURV	NGL	NCL	RF	SL	NRP	ARL	b
CONMA	–								–
SURV	–0.99**	–							–0.58
NGL	–0.97**	0.97**	–						–8.72
TNL	–0.99**	0.99**	0.99**	–					–7.74
RF	–0.96**	0.92**	0.94**	0.93**	–				–o.32
SL	–0.97**	0.93**	0.92**	0.93**	0.99**	–			–8.64
NRP	–0.48*	0.51*	0.51*	0.50*	0.40^{NS}	0.33^{NS}	–		–13.73
ARL	–0.51*	0.53*	0.51*	0.51*	0.44^{NS}	0.40^{NS}	0.98**	–	–9.29

* = Significant at p = 0.05 probability level, ** =Significant at p = 0.01 probability level, NS = not significant. CONMA = concentration of mannitol, SURV = Survival of explants, NCL = Number of chlorotic leaves, NGL = Number of green leaves, SL = Shoot length, RF = Root formation, NRP = Number of root per plantlet, ARL = Average root length per plantlet, b = regression coefficient.

leaves was produced in medium free of mannitol, while the highest number of leaves was produced at 5 and 10 g/l mannitol in MV 99/0395 and TMS 01/1206, respectively (Table 4). Reduction in number of leaves under drought conditions might be for the purpose of decreasing transpiration and adjustment of photosynthetic machinery, as leaves are the organs that contain chloroplasts and stomata, which are associated with photosynthesis and transpiration, respectively. According to Roitsch (1999), when water supply is significantly decreased, plants adjust growth, leaf formation and photosynthetic activities, which affect carbon partitioning between tissues that serve as sink and source. As a result, sugars that are utilized for normal plant growth are redirected to selective growth of roots and shoots or towards production of osmoprotectants (Lei et al., 2006). In the present study, leaves were sensitive to mannitol-induced drought stress, as all cultivars exhibited reduced number of leaves to a varying degree at 25 and 30 g/l mannitol. No cultivars showed an increase in number of leaves as an adaptive strategy. In TMS 01/1206, number of roots produced decreased as the concentration of mannitol in culture media increased, whereas in CH140, number of roots increased as the concentration of mannitol increased before decreasing; while in MV 99/0395, number of roots was not affected by an increase in mannitol concentration. Besides anchorage, the main function of roots is water and mineral absorption. Hence, root proliferation was used as an drought adaptive strategy by cultivar CH140 in this study to expand search for water and mineral elements under mannitol-induced drought. The average root length was longest at 5 and 10 g/l mannitol in CH 140 and TMS 01/1206 while there was no difference in average root length of MV 99/0395 among different mannitol concentrations. To avoid drought stress and disruption of metabolic and physiological activities at mild drought stress, cultivars CH 140 and TMS 01/1206 might increase root length to expand surface contact with soil to enhance water uptake. Similar findings have been reported on some other cultivars of cassava (El-Sharkawy and Cadavid, 2002; Aina et al., 2007). The highest number of shoots was observed in TMS 01/1206 at 5 and 15 g/l mannitol, in CH 140 at 15 and 20 g/l mannitol, and in MV 99/0395 at 5 and 10 g/l. Formation of additional shoots at different level of drought stress could be related to water stress coping strategy of the three contrasting varieties. This could be a mechanism to expand surface area for photosynthesis and serve as temporary storage for water. Morphological responses of cassava to drought stress are in various ways according to El-Sharkaway (2012); one of which is formation of additional shoots to serve as temporary storage of available water and to enhance light-harvesting capacity; hence, the observed increased number of shoots in the above-noted five cultivars.

Relationships between growth parameters and mannitol concentration

Concentration of mannitol had significant negative correlation with all parameters at 1% except NRP and ARL (Table 5). Survival of explants in culture medium had significant positive correlation with NGL, NCL, RF and SL at P<0.01 and NRP and ARL at $P < 0.05$ (Table 5). Similarly, NGL had significant positive correlation with NCL, RF and SL at $P < 0.01$ and NRP and SL at $P < 0.05$. The NCL had significant positive correlation with RF and SL at $P < 0.01$ and NRP and ARL at $P < 0.05$. However, RF only had significant positive correlation with SL at $P < 0.01$. The NRP had significant positive correlation with ARL at $P < 0.01$. Coefficients of regression of growth parameters on concentration of mannitol were negative and ranged from –0.32 to –13.73 (Table 5). The lowest b were recorded by SURV and RF while the highest b was observed in NRP. Linear correlation analysis revealed two forms of relationship in this study: first, the relationship among growth parameters and second, the relationship between concentration

of mannitol and growth parameters. The two forms of relationship have applications in *in vitro* screening of cassava for drought tolerance using mannitol-induced stress (Neto et al., 2004). In the present study, survival of explant and number of green leaves per plantlet had strong, positive and significant correlation with other growth parameters suggesting that other growth parameters could realistically be estimated from either of the two in a large scale *in vitro* screening experiments to identify drought tolerance varieties to save time. The relationships between concentration of mannitol and growth parameters were negative with minimum magnitude (as revealed by regression coefficient) on survival of explant and rooting frequency, which is consistent with findings of Hassanein (2010), has implications on determination of the optimum concentration of mannitol for inducing water stress in wide diverse of cassava germplasm.

CONCLUSION

The three varieties expressed differential response, based on their sensitivity to drought stress from the presence of mannitol (drought -inducing agent) in the culture medium. Variety CH140 had an outstanding performance in terms of explants survival, shoot length, number of green leaves, average root length and frequency of root formation which was informed by drought tolerant ability of the variety. Our results, therefore, suggested that mannitol is a suitable *in vitro* drought inducing-agent for screening cassava varieties for drought tolerance. Based on survival of explants in culture medium, 20 g/l mannitol is the best concentration for cassava *in vitro* screening.

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