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Effects of Auxin and Source of Explants on Callus Induction of Tropical Maize

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Abstract: Induction of callus from explants is a critical process in regeneration, micropropagation and transformation of plants. Formation of callus from plant tissues on culture is affected by different factors. This study sought to establish the effect of genotype, source of explants and auxin concentration on callus induction from different Sudanese maize genotypes (222F, Hudiba-1, 441, Giza-2, PR5655 and Mojtamma-45). Callus induction of the six maize varieties was investigated using mature embryos, leaf disks and shoot tips as explants and different concentrations of the auxin; 2,4-dichlorophenoxyacetic acid (2,4-D), ranging from 0 to 10 mg L^{-1}. The highest callus, induction frequency was observed in shoot tips while the lowest was observed in mature embryos. Leaf disks gave a higher callus induction frequency than mature embryos and lower than shoot tips. Concentrations of 2,4-D of 2 mg L^{-1} gave the highest callus induction for most genotypes while 0 and 10 mg L^{-1} gave the lowest callus induction for all the genotypes.

Key words: 2,4-D, tropical maize, mature embryos, leaf disks, shoot tips, callus

INTRODUCTION

Maize is an important grain crop used as human food, feed for animals and an important source of products such as sweeteners, starch, alcohol and oil (Ahsan *et al*., 2000). It has become localized staple food in sub Sahara Africa, providing 50% of the basic calories (Machuka, 2001) and an important source of carbohydrate, protein, iron, vitamin B and minerals. In Sudan maize is produced using traditional or mechanical methods and is mainly used for food, forage and is a potential source of foreign exchange through export (Omer *et al*., 2008). Maize production is affected by abiotic and biotic factors. Biotic factors include weeds such as *Striga* and insects. Regionally, low yield is a major factor that affect maize crop leading to lack of adequate food and repeated spells of hunger, malnutrition and related deficiency diseases among the population. Alternative demands for maize for animal feed and bio fuel has increased the shortage of maize for food due to the better price provided by industrial users.

In Sudan the most serious biotic factors affecting maize yield are weeds such as *Striga* and insects such as stem borer (FAO, 2000). Abiotic factors include low soil fertility, high temperatures and drought. The main abiotic factor constraining maize production in the sub Sahara Africa is drought, estimated to cause an annual loss of 17% in maize production, with up to 70% loss under severe conditions (Edmeades *et al*., 1995). Other important abiotic factors include low soil fertility and high temperatures. These constraints can be overcome through development of varieties that can tolerate or resist the stresses. This can be done though complementing conventional breeding and genetic transformation. Success in plant transformation is dependent on the ability to regenerate a whole plant from transformed tissues or cells (Ahmadabadi *et al*., 2007).

Most of the previous studies on tissue culture of maize has been achieved using temperate maize genotypes (Green and Philips, 1975; Negrotto *et al*., 2000). It was only very recently that further studies were undertaken to evaluate the response of more tropical maize genotypes to tissue culture (Ombori *et al*., 2008; Oduor *et al*., 2006; Omer *et al*., 2008; Matheka *et al*., 2008). Through tissue culture approach, describable qualitative and quantitative agronomic traits have been achieved in crops (Machuka, 2001). In this study, six Sudanese maize genotypes were evaluated for their response to tissue culture at different auxin concentrations using three different explant sources.

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MATERIALS AND METHODS

Plant materials: Seeds of maize varieties, 222F, Hudiba-1, 441, Giza-2, PR5655 and Mojtamma-45, were sterilized by first soaking in 70% ethanol for 30 sec, then in 2.5% sodium hypochlorite for 30 min before washing with sterile water three times (Fig. 2a). The sterile seeds were used as sources of mature embryos as explants for callus induction. To establish plants to obtain shoot tips and leaf disks explants, sterile seeds were planted in sterile jam jars (Fig. 1a, 3a) containing MS basal salts (Murashige and Skoog, 1962). The jars were kept in a growth room and maintained at a temperature of 28°C and 16/8 h light/dark photoperiod (100 μmol m^{-2} sec^{-1}).

Preparation of callus induction media and culture conditions: All callus induction experiments were performed on Callus Induction Medium (CIM) medium comprising of MS salts and vitamins supplemented with 2.875 g L^{-1} L-proline, 0.1 g L^{-1} casein hydrolysate, 10 mg L^{-1} silver nitrate and 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 with 1 M NaOH or 0.1 M HCl and 0.8% (w/v) agar added before autoclaving to sterilize. The sterilized medium was allowed to cool before adding 2,4-D. The medium was dispensed in sterile petri dishes in volumes of 30 mL and allowed to solidify. Explants were then cultured on the medium and plates sealed with parafilm (Fig. 1b, 2b, 3b).

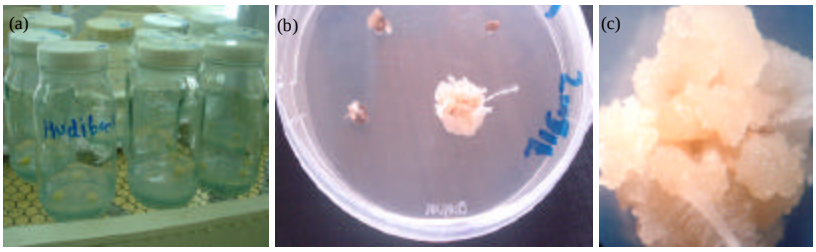

Fig. 1(a-c): Induction of callus from shoot tips of tropical maize achieved using 2,4-D, (a) Maize plants germinated aseptically as source of shoot tip explants, (b) Callus induced from shoot tips on CIM containing 2 mg L^{-1} 2,4-D and (c) Appearance of a callus induced from a shoot tip explant on CIM containing 2 mg L^{-1} 2,4-D

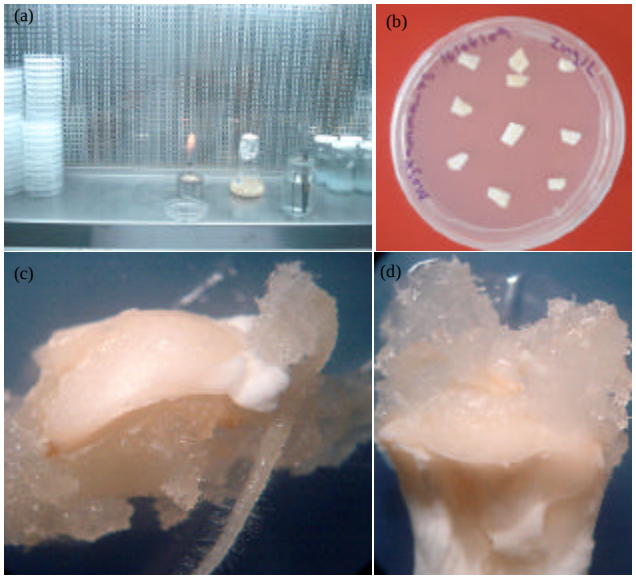

Fig. 2(a-d): Induction of callus from mature embryos of tropical maize achieved using 2,4-D, (a) A setup of aseptic working conditions for excision of mature embryos from sterilized maize seeds, (b) Mature embryos cultured on CIM containing 2 mg L^{-1} 2,4-D and callus induced from mature embryos of (c) 222F and (d) Hudiba-1 genotypes

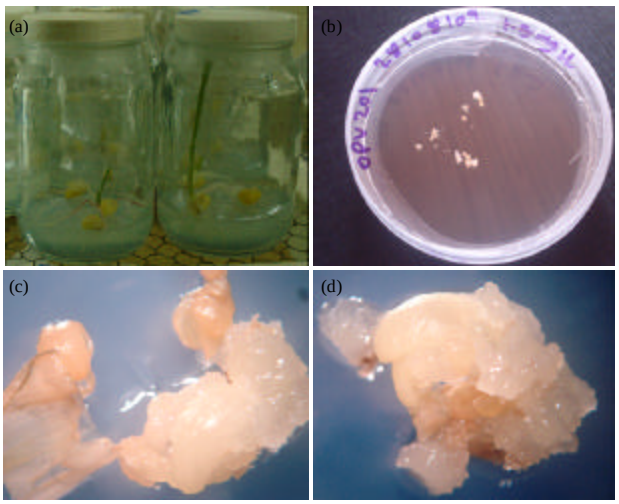

Fig. 3(a-d): Induction of callus from leaf disks of tropical maize genotypes achieved using 2,4-D, (a) Sterile germination of maize plants to serve as a source of leaf disk explants, (b) A culture of leaf disk explants on CIM, containing 1.5 mg L^{-1} 2,4-D for induction of callus and callus induced from leaf disks of (c) Giza-2 and (d) Mojtamma-45 genotypes

Ten levels of 2,4-D (0, 0.5, 1, 1 .5, 2, 2.5, 3, 3.5, 4, 10 mg L^{-1}) were tested to establish their efficacy in establishing callus from three different explants via mature embryos, leaf disks and shoot tips.

The mature embryo explants were obtained by removing the seed coat from sterile seeds and then soaking in sterile distilled and autoclaved water over night. Mature embryos were excised using a sterile scalpel blade and cultured in CIM containing different 2,4-D concentrations. The embryos were orientated with the embryo axis in contact with the medium. Twenty embryos were cultured in a 90×15 mm Petri dish for each 2,4-D level. The cultures were incubated in the dark at 27±1°C for two weeks. Callus induction frequency was recorded after four weeks of culture on the CIM.

The shoot tips explants were excised from three week old plants and cultured on CIM containing the different 2,4-D concentrations with twenty shoot tips for each concentration. Callus induction frequency was calculated after one month of culture. The leaf disk explants (1 cm^2) were excised from 3 week old plants (Fig. 3a) and cultured on CIM for three weeks before calculating callus induction frequency.

Statistical analysis: The experiments were designed in RCBD with four replications per treatment. Callus Induction Frequency (CIF) was calculated as number of callus induced per the total number of explants cultured and expressed as a percentage. Analysis of variance (ANOVA) was done using StatView statistical software to test the statistical significance of differences among explants source and 2,4-D levels. Mean separation was done using Least Significance Difference (LSD) test at 5% probability level.

RESULTS

Different levels of 2,4-D were found to cause different responses in callus induction from the three types of explants for each genotype. Results in Table 1 show that callus induction frequency from the shoot tips of the genotype 222F was highest at 2,4-D concentrations of 2 and 2.5 mg L^{-1} (7.58±0.31 and 7.05±0.29%, respectively). The two concentrations were not significantly different from each other but were significantly different from the other concentration in callus induction. The same levels of 2,4-D (2 and 2.5 mg L^{-1}) gave the highest CIF from leaf disk of 222F (7.55±0.52 and 6.50±0.20%), which was significantly higher than the other 2,4-D levels. However, no significant difference was found between the two concentrations. There were no significant differences in callus induction at concentrations of 2.5 and 3.5 mg L^{-1} of 2,4-D (Table 2). Mature embryos of 222F were observed to start calling from between 3 to 4 weeks (Fig. 2c). For mature embryos, the highest CIF was obtained at 2,4-D concentration of 3 mg L^{-1} with a mean of 3.43±1.20%. This level was not significantly different from 2, 2.5, 3.5 and 4 mg L^{-1} of 2,4-D in CIF (Table 3). However, it was significantly higher than the rest of 2,4-D concentrations.

Table 1: Callus induction from shoot tips

2,4-D conc. (mg L^{-1})	222F	Hudiba-1	441	Giza-2	PR5655	Mojtamma-45
0.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.50±0.20	0.00±0.00
0.5	0.80±0.80	3.56±0.33	1.60±0.92	3.20±0.00	7.55±0.15	6.30±0.33
1.0	0.80±0.80	5.45±0.37	4.75±0.25	6.70±0.23	6.90±0.20	7.20±0.33
1.5	4.18±0.33	6.10±0.20	4.50±0.00	7.55±0.15	8.53±0.13	7.70±0.00
2.0	7.58±0.31	8.65±0.14	9.05±0.15	9.48±0.23	8.48±0.28	8.78±0.13
2.5	7.05±0.29	8.30±0.35	6.50±0.20	8.05±0.20	9.48±0.23	7.40±0.17
3.0	4.68±0.554	6.90±0.20	5.00±0.29	5.25±0.25	10.00±0.00	6.70±0.23
3.5	4.75±0.25	5.90±0.40	4.75±0.25	4.75±0.25	7.40±0.17	6.10±0.20
4.0	3.85±0.375	5.25±0.25	3.85±0.38	4.50±0.00	6.70±0.23	5.50±0.00
10.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.90±0.20	0.00±0.00
LSD	1.256	0.779	1.055	0.482	0.553	0.547
p-value	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001	≤.0001

Table 2: Callus induction from leaf disks

2,4-D conc. (mg L^{-1})	222F	Hudiba-1	441	Giza-2	PR5655	Mojtamma-45
0.0	0.00±0.00	0.00±0.00	0.80±0.80	0.00±0.00	5.00±0.29	0.00±0.00
0.5	0.00±0.00	0.80±0.80	2.50±1.46	2.50±1.46	7.55±0.15	0.00±0.00
1.0	0.80±0.80	4.50±0.00	4.68±0.55	4.43±0.47	6.90±0.20	0.80±0.80
1.5	2.73±0.96	6.10±0.20	5.00±0.29	5.25±0.25	8.60±0.30	7.70±0.00
2.0	7.55±0.52	8.75±0.38	5.90±0.40	5.20±0.44	9.75±0.14	7.25±0.15
2.5	6.50±0.20	7.90±0.32	5.40±0.61	4.68±0.55	7.58±0.31	7.25±0.15
3.0	4.75±0.25	8.23±0.18	4.18±0.33	3.85±0.38	9.75±0.13	4.95±0.45
3.5	5.90±0.23	6.70±0.23	3.15±1.82	2.50±1.46	6.50±0.38	5.40±0.52
4.0	4.18±0.33	4.75±0.25	2.50±1.46	0.00±0.00	6.90±0.20	3.05±1.62
10.0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
LSD	1.390	0.854	2.747	2.180	0.823	1.362
p-value	≤.0001	≤.0001	≤.0014	≤.0001	≤.0001	≤.0001

Table 3: Callus induction from mature embryos

2,4-D conc. (mg L^{-1})	222F	Hudiba-1	441	Giza-2	PR5655	Mojtamma-45
0.0	0.00±0.00	0.80±0.80	2.93±0.91	0.00±0.00	0.00±0.00	0.00±0.00
0.5	0.00±0.00	0.00±0.00	2.23±0.56	0.80±0.80	2.23±0.56	0.00±0.00
1.0	1.93±0.78	0.00±0.00	2.60±0.77	2.93±0.91	2.23±0.56	0.00±0.00
1.5	1.10±0.76	3.43±1.20	3.18±1.08	3.18±1.08	2.23±0.56	3.43±1.22
2.0	2.23±0.56	3.18±1.08	2.93±0.91	2.93±0.91	2.60±0.77	2.93±0.91
2.5	2.93±0.91	1.10±0.76	3.43±1.20	3.43±1.20	0.00±0.00	2.93±0.91
3.0	3.43±1.20	1.13±0.76	2.55±0.80	2.55±0.80	0.00±0.00	2.55±0.80
3.5	2.93±0.91	0.00±0.00	2.93±0.91	2.23±0.56	0.00±0.00	1.10±0.76
4.0	2.93±0.91	0.00±0.00	2.93±0.91	2.93±0.91	0.00±0.00	0.00±0.00
10.0	0.00±0.00	0.00±0.00	2.93±0.91	2.23±0.56	0.00±0.00	0.00±0.00
LSD	1.479	1.74	2.630	1.214	0.946	1.895
p-value	≤.0001	≤.0001	0.9983	≤.0001	≤.0001	≤.0001

The highest callus induction from shoot tips of Hudiba-1 of 8.65±0.14 and 8.30±0.35% was observed at 2,4-D concentrations of 2 and 2.5 mg L^{-1}, respectively. These 2,4-D levels were significantly higher than all the other 2,4-D concentrations in terms of CIF. However, there was no significant difference between these two concentrations in induction of callus from Hudiba-1 shoot tips (Table 1). Callus induction from Hudiba-1 leaf disks was highest at 2 and 3 mg L^{-1} 2,4-D concentrations with mean CIF of 8.75±0.38 and 8.23±0.18%, respectively (Table 2). Figure 2d shows callus induced on Hudiba-1 mature embryos 28 days after culturing on 2,4-D containing CIM. The highest CIF from Hudiba-1 mature embryos were 3.43±1.20 and 3.18±1.08% obtained with 2,4-D concentrations of 1.5 and 2 mg L^{-1}, respectively. These concentrations were significantly higher than the other concentrations of 2,4-D in CIF, but were not significantly differences from each other (Table 3).

For the genotype 441, 2 mg L^{-1} 2,4-D was observed to produce the highest CIF (9.05±0.15%) on shoot tip explants. This 2,4-D level gave a significantly higher CIF than that obtained by using the other levels of 2,4-D (Table 1). The highest callus induction from 441 leaf disks was 5.90±0.40 and 5.40±0.61%, obtained at 2,4-D concentrations of 2 and 2.5 mg L^{-1}, respectively (Table 1). These two 2,4-D levels were not significantly different from each other but were significantly higher than 0, 1, 4 and 10 mg L^{-1} in CIF. The highest callus induction frequencies from mature embryos of genotype 441 were 3.18±1.08 and 3.43±1.20% obtained by culturing at 2,4-D concentrations of 1.5 and 2.5 mg L^{-1}, respectively.

Interestingly, there was no significant differences in callus induction between these two concentrations and the other 2,4-D concentrations (Table 3).

Shoot tip explants from Giza-2 produced callus at 2 and 2.5 mg L^{-1} 2,4-D levels at the highest CIF of 9.48±0.23 and 8.05±0.20%, respectively (Table 1). Callus induction at this concentration was significantly higher than at other 2,4-D concentrations.

Leaf discs of Giza-2 proliferated callus more easily and quickly than leaf disks of other genotypes. It took between 10-15 days for callus to emerge (Fig. 3c) and 25 days for complete conversion of the explants into callus.

This genotype gave the highest callus induction on leaf disks of 5.25±0.25 and 5.20±0.44% at a 2,4-D concentrations of 1.5 and 2 mg L^{-1}, respectively. However, no significant difference between the two 2,4-D concentrations in callus induction were observed. Additionally, the two levels gave significantly higher CIF than the other 2,4-D concentrations (Table 2).

The highest callus induction from mature embryos of Giza-2 was observed at the 2,4-D concentrations of 1.5 and 2.5 mg L^{-1} which produced CIF of 3.18±1.08 and 3.43±1.20%, respectively. There was no significant difference in callus induction between the two 2,4-D concentrations. However, the two levels were significantly higher than the control (0 mg L^{-1}) and 0.5 mg L^{-1} 2,4-D level. Concentrations of 1.5 and 2.5 mg L^{-1} produced superior callus induction frequency compared to the other levels of 2,4-D though the differences were not significant (Table 3).

Cultured shoot tips obtained from 4-week old PR5655 plants produced callus on CIM medium containing 2,4-D (Fig. 1b). The callus can be described as white and embryogenic in appearance. The callus was observed to have the tendency of producing roots on the CIM (Fig. 1c). The highest callus induction of 9.48±0.23 and 10.00±0.00% observed from shoot tips of the genotype PR5655 was obtained at 2,4-D concentrations of 2.5 and 3 mg L^{-1}, respectively. These CIFs were significantly higher compared to these produced by the other concentrations of 2,4-D. However, but there was no significant differences observed between the two level in response to CIF. The leaf disks gave the highest callus induction frequency of 9.75±0.14 and 9.75±0.13 from 2,4-D concentrations of 2 and 3 mg L^{-1} (Fig. 3). The highest callus induction frequency from mature embryos was 2.60±0.77 from 2,4-D concentrations of 2 mg L^{-1} This was significantly higher than the other 2,4-D concentrations but there was no significant differences between 2 and 0.5 mg L^{-1} as well as 1 and 1.5 mg L^{-1}.

The 2,4-D concentrations of 1.5 and 2 mg L^{-1} produced the highest CIF of 7.70±0.00 and 8.78±0.13% from shoot tips excised from Mojtamma-45. These levels were significantly higher than the other 2,4-D concentrations in CIF. In addition, the 1.5 mg L^{-1} 2,4-D level did not differ significantly from 1 mg L^{-1} 2,4-D in CIF (Table 1). Leaf discs from Mojtamma-45 were observed to swell and fold slightly before forming callus on 2,4-D-containing CIM (Fig. 3d). The callus was cream colored and watery in appearance. The highest callus induction from leaf disks was 7.70±0.00% obtained at a 2,4-D concentration of 1.5 mg L^{-1}. This was significantly higher compared to callus induction at other 0, 0.5, 1, 3.0, 3.5, 4, 4.5 and 10 mg L^{-1} 2,4-D concentrations. However, it was not significantly different from 1.5, 2.0 and 2.5 mg L^{-1} 2,4-D levels in CIF (Table 2). The highest callus induction from mature embryos was 3.43±1.22%, obtained at a 2,4-D concentration of 1.5 mg L^{-1} and was significantly higher than at the other tested 2,4-D concentrations (Table 3).

DISCUSSION

Standard protocols for tissue culture for temperate maize were first developed by Green and Philips (1975). Subsequently many studies have emerged reporting different responses of maize to tissue culture depending on the source of explants and the concentrations of auxin used. Protocols for efficient induction of callus from different tissues of tropical maize have been developed in this study.

In our experiments we noticed that different amounts of 2,4-D used in the culture medium promoted different responses to callus induction by the explants used. For example the shoot tips from all the genotypes tested formed callus at highest frequency when exposed to 2 and 2.5 mg L^{-1} of 2,4-D. This was true for Mojtamma-45 except that 1.5 mg L^{-1} 2,4-D also induced callus as efficiently as the 2 and 2.5 mg L^{-1}. Present findings are in agreement with those reported by Muoma *et al*. (2008) where the 2,4-D concentration of 2 mg L^{-1} in combination with BAP induced organogenic callus efficiently from shoot tips of some tropical maize varieties. Additionally a fast, simple and efficient system for production of callus from shoot tips from tropical and subtropical lines was described by O'Connor-Sanchez *et al*. (2002).

The leaf disc explants from four genotypes (441, Giza-2, PR5655 and Mojtamma-45) induced callus most efficiently in the presence of 1.5 and 2 mg L^{-1} 2,4-D. However, 2 and 2.5 mg L^{-1} of 2,4-D was sufficient to form callus from the other two genotypes (Hudiba-1 and 222F) most efficiently. Callus induction from leaves was also

reported by Ahmadabadi *et al*. (2007). They noted plant regeneration from callus induced from leaf discs is a cost effective alternative to currently available maize tissue culture methods as it requires less labour and time.

We noticed that 2,4-D was required at low concentrations for induction of callus from mature embryos for all the genotypes evaluated. All the genotypes induced callus most efficiently at 1.5 mg L^{-1} 2,4-D except 222F which required 2,4-D at 2 mg L^{-1} for high callus induction. Interestingly the mature embryos from genotypes 441 and PR5655 induced callus efficiently at very low 2,4-D concentrations of 0 and 0.5 mg L^{-1}, respectively. However, further increase in 2,4-D level had no noticeable effect in callus induction from PR5655 and Hudiba-1 beyond 2 and 3 mg L^{-1}, respectively. According to Rakshit *et al*. (2010) and Manivannan *et al*. (2010) the presence of 2,4-D in the culture medium is important for cereal callus induction and callus formation. Mature embryos from dry seeds are more readily available and can be used as an effective and alternative explants source in tissue culture of maize (Al-Abed *et al*., 2006; Abebe *et al*., 2008).

We conclude that callus induction from different maize tissues is dependent on the amount of the 2,4-D used. We recommend use of the reported CIM supplemented with 2-2.5 mg L^{-1} 2,4-D and 1.5-2 mg L^{-1} 2,4-D for efficient induction of callus from shoot tips and leaf discs, respectively, obtained from the reported genotypes. For callus induction from mature embryos, 1.5 mg L^{-1} 2,4-D is recommended although lower concentrations may be evaluated further for the reported genotypes.

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