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The Direct Regeneration of Maize Haploids Through Anther Culture

[1]L. Hassan, [2]S.D. Ahmad and [3]A. Okumus
[1]Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, [2]University College of Agriculture, Rawalakot Azad Kashmir, Pakistan, [3]Ondokuzmayis Universitesi, Ziraat Fakultesi, Turkiye

Abstract: The genetics of plants regenerated from anther-derived callus was investigated using single crosses & inbreds of maize. Anther culture in maize (*Zea mays* L.) usually follows an indirect procedure involving callus induction and differentiation on at least two different media. Alternatively, development of a direct procedure by which plantlets can be regenerated directly on one medium seems desirable, if acceptable frequency of plant regeneration could be obtained and an effective procedure of chromosome doubling of regenerated plantlets is available. Yupie (YP) and N6 were used as basal media and 10 growth regulators (2,4-D, kinetin, NAA, IAA, IBA, dicamba, pCPA, 6-BA, 2ip and hypoxanthine) were tested to evaluate the feasibility of direct regeneration of haploid plantlets of eight single cross hybrids and four inbred lines. The results indicated that the direct generation of plantlets is feasible through anther culture, though it is genotype and medium dependent. Ten plantlets were regenerated directly from single cross hybrid M60 X M96. The more effective medium was N6 basal medium plus 2,4-D (2.0 mg l^{-1}) and kinetin (1.5 mg l^{-1}). Regeneration frequency (percent of anthers producing plantlets) ranged from 1 to 4%.

Key words: Haploid, anther culture, maize, *Zea mays* L.

Introduction

Maize is a crop with great adaptability originally from the tropics, its range has been continuously extended into temperate zones through breeding and selection. However, there is a need for wider genetic diversity. Anther culture is an excellent system for investigation of genetic recombination through gamete analysis and gametoclonal variation in pure lines (Hu, 1989). Cell and tissue culture techniques are already used in breeding process of several plant species. In maize, regeneration via anther culture is one of the most promising techniques to speed up the breeding progress (Petolino and Jones, 1986). This versatile technique has not been used on a large scale so far, mainly due to the limited regenerability of maize. Anther culture ability can be improved by further optimization of culture methods.

In the development of pure lines of maize production of haploid through anther culture is considered a useful alternative approach (Kuo *et al.*, 1986). However, in general, an indirect or two step procedure is used in maize anther culture, i.e., callus induction is carried out in one medium and the differentiation in the other. Alternatively, development of a direct regeneration or one step procedure whereby plantlets can be produced directly from anthers on one medium may be more efficient in terms of labour, time space, and materials compared to an indirect approach. Such a procedure seems feasible because a direct procedure has been successful in tobacco (*Nicotiana tabacum* L.) (Nitsch and Nitsch, 1969) and wheat (*Triticum aestivum* L.) (Liang *et al.*, 1987), because maize anthers are less sensitive to hormones than other plant species (Chen *et al.*, 1979; Ku *et al.*, 1977; Kuo *et al.*, 1986; Tsay *et al.*, 1986). Direct regeneration of haploid corn plantlets on an induction medium has been suggested by Chen *et al.* (1979). The aim of this study was to determine if direct generation of haploid maize plantlets was feasible using known media supplemented with different growth regulators at various concentrations.

Materials and Methods

Plant materials included 8 single-cross hybrids, and 4 inbred lines. Single crosses used for *in vitro* anther culture were: M60 x M96, M95 x S35, M86 x S35, M95 x FERKE, M71 x M72, M68 x M77, M75 x M80 and M85 x M91 and inbred lines were: S2-10, S21-8 and S53-1. Basal media used were N6 (Chu *et al.*, 1975) and YP (Ku *et al.*, 1977; Genovesi and Collins, 1982) with various combinations of growth regulators as bellow.

1. N6 + kinetin (1.5mg l^{-1})
2. N6 + 2, 4-dichlorophenoxy acetic acid (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
3. N6 + ∝-naphthalene acetic acid (NAA) (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
4. N6 + indol-3-acetic acid (IAA) (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
5. N6 + indol-3 butyric acid (IBA) (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
6. N6 + dicamba (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
7. N6 + p-chlorophenoxy acetic acid (pCPA) (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
8. N6 + NAA (2mg l^{-1}) + 6-benzyladenine (6-BA) (1.5 mg l^{-1})
9. N6 + NAA (2 mg l^{-1}) + 6 γ,γ-dimethylallylamino)-purine (2ip) (1.5 mg l^{-1})
10. N6 + NAA (2mg l^{-1}) + hypoxanthine (1mg l^{-1})
11. YP + kinetin (1.5 mg l^{-1})
12. YP + 2,4-D (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
13. YP + NAA (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
14. YP + IAA (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
15. YP + IBA (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
16. YP + dicamba (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
17. YP + pCPA (2 mg l^{-1}) + 6-BA (1.5 mg l^{-1})
18. YP + NAA (2 mg l^{-1}) + kinetin (1.5 mg l^{-1})
19. YP + NAA (2 mg l^{-1}1) + 2-ip (1.5 mg l^{-1})
20. YP + NAA (2 mg l^{-1}) + hypoxanthine (1 mg l^{-1})

Florets with anthers containing microspores at the uni-nucleate stage were collected prior to anthesis and sterilised by submerging in 0.2% commercial bleach for 10 minutes and then washed five times with sterile distilled water. Forty anthers were then placed on 25ml of medium in 100 X 20 mm disposable petri dishes. The petri dishes were kept in an incubation chamber at 28 °C under darkness until there were signs of callus or embryoid formation. They were then transferred to a chamber with 12 h light and light intensity of

Table 1: Frequencies of callus and embryoid formation for 8 responding corn hybrids and inbreds on YP and N6 media

Genotypes	Induction frequency per 100 anthers	
	N6	YP
Hybrids		
M60 x M96	8.6	16.3
M95 x S35	4.1	6.8
M86 x S53	0.0	5.1
M95 x FERKE	0.0	2.9
Inbreds		
S2-9	0.8	1.9
S2-10	0.0	5.6
S21-8	0.0	3.0
S52-1	0.0	2.9

60 Es^{-1} m^{-1} at 18 ^{0}C. When roots and shoots were formed , the seedlings were transferred to the hormone free N6 basal medium/vermiculite. As a whole, the experiment was replicated for three times.

Regenerated seedlings were grown in vermiculite transferred to pots in a greenhouse after the formation of 2 to 4 leaves and 2 to 3 roots. Root tips were taken at the time of transplantation and pre-treated in distilled water at 1 ^{0}C for 24 h and fixed in Cornoy B solution (6 "95%" EtOH: 3 chloroform: 1 acetic acid) at 4 ^{0}C until slide preparation. Enzyme treatment of root tips and slide preparations were made according to a similar procedure of Song *et al.* (1988).

Results and Discussion

Forty days after placing anthers on the media, initiation of calli and embryo-like structure (embryoid) occurred and continued for another 30 days. The majority of responding anthers produced embryoids which were potentially regenerable, whereas calli failed to redifferentiate. The surface structures of the embryoids were smooth and globular in shape while calli were generally rough on the surface and irregular in shape.

Initiation of calli and embryoids on those two media appeared to depend on genotypes. Among 12 genotypes tested, 8 produced calli and embryoids with various frequencies (Table 1). The results were availed by taking the mean values of three replications. However, the differences between the replications were not found to be much larger, calli and embryoids were some times produced simultaneously by a genotype on the same medium. The induction frequency of calli and embryoids ranged from 2.9 to 16.3 per 100 anthers plated. The highest frequency, being 16.3% occurred for single cross hybrid M60 x M96. YP medium was more effective than N6 in callus and embryoid induction. More than seventy percent of the calli and embryoids were produced on YP medium whereas on N6 medium the response of maize hybrids was 50% but it was less than 50% in case of inbreds.

Organ formation from embryoids occurred about 20 days after their initiation. However, many had only root-like structures but no shoots, some developed shoots but no roots, and others failed to differentiate and died later. Direct formation of plantlets only occurred for hybrid M95 x S95 and inbred S2-9 on N6 medium supplemented with 2, 4-D (2 mg l^{-1}) and kinetin (1.5 mg l^{-1}). Such variable genotypic response is very common in *in-vitro* culture. A total of 13 plantlets were obtained and chromosome counts of the plantlets confirmed that these were haploids (unpublished data).

Although the frequency of haploid production was low, yet it indicated that the haploid production is possible using a direct regeneration approach. Having bipolar structures with root and shoot meristems those somatic embryoids had similar structural organizations as the zygotic embryos. Embryoids had much higher potential for regeneration than calli (Cheng *et al.*, 1987). Since many embryoids produced roots without shoots or vice versa, it has been suggested that a balance of auxin and cytokinin is necessary for simultaneous formation of roots and shoots (Ke, 1987).

Since plantlet regeneration occurred primarily through embryogenesis in both indirect and direct anther cultures, it is conceivable that the entire path of plantlet development can be achieved by a one step procedure. However, problem remains for the practical use of a direct plantlet regeneration approach. Factors related to high regeneration frequency, such as genotype, pre-treatments and incubations must be analyzed. Also, effective chromosome doubling of the embryoids and calli rather than the seedlings, as reported by Wan *et al.* (1989) should be evaluated.

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