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Full Length Research Paper

Screening tropical maize lines for the production and regeneration of friable and embryogenic type II callus

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Twenty two maize lines popularly used by breeders in the Kenyan maize program were screened for their ability to form type II callus. The immature embryos were harvested two weeks after pollination. Two highland lines showed high embryogenic response and formed friable, type II calli. While 16 lines screened showed poor or retarded growth with subsequent death. There was successful plant regeneration from four lines collected from the highland breeding program that are genetically related. The results suggested that genetic factors may be responsible for the differences seen in the regenerability of the tropical lines. The two lines CMB7 and CMB8 are considered suitable for further evaluation using a transformation system

Key words: Tropical maize lines, immature embryos, embryogenic callus, regeneration.

INTRODUCTION

The ability to regenerate maize embryo derived from callus cultures has been reported to be dependent on the genotype used (Fluminhan and Aguiar-Perecin, 1998; Lee and Phillips, 1987). The first report on plant regeneration from maize embryo-derived callus cultures was by Green and Phillips (1975). In later years regeneration of several inbreds adapted to tropical and subtropical regions have been shown to produce embryonic calli (Santos-Serejo and Aguiar-Perecin, 2000; Bohorova et al., 1995; Furini and Jewell, 1994; Walters et al., 1992; Prioli and Silva, 1989). There are several reports on the regeneration of inbreds and hybrids adapted to temperate regions, many of these being type I callus (Sidorov et al., 2006; Fluminhan and Aguiar-Perecin, 1998). Usually the calli are derived from precultured immature embryos (Ishida et al., 1996; Songstad et al., 1996). Regeneration and transformation of temperate lines with A*grobacterium tumefaciens* has been widely reported. However successful reports on transformation of tropical maize lines are limited. This may be due to the difficulties in identifying tropical lines with the ability to regenerate plants from precultured immature embryos. An important step would therefore be the production of callus capable of plant regeneration from immature embryos (Duncan et al., 1985). Bohorova et al. (1995) reported several successes of embryogenic calli and plant regeneration obtained from 50% of tropical and subtropical lines, 87% of mid-altitude lines and 75% of highland lines tested. The lines were later used in the transformation of maize for *Bacillus thuringiensis* (*Bt*) resistance using microprojectile bomb-ardment (Bohorova et al., 1998).

The objective of this work was to identify tropical maize lines commonly used by breeders for plant regeneration from callus formed using immature embryos with the aim of using successful lines for *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

Plant material

Twenty two inbred lines collected from breeding programs in different ecological zones in Kenya were used. These lines are commonly used by breeders to make hybrids for different parts of the country. They have different maturation period and the sampling was staggered according to the pollination dates. The composition of the lines was as follows: CIMMYT (international maize and wheat improvement centre) lines; CML202, CML395, CML442, CML444, POOL A6-3; Kenyan breeder lines designated as follows: highland lines: CMB7, CMB8, CMB9, CMB17, CMB19, CMB20 and CMB21; mid-altitude lines: CMB10, CMB11, JI15, CMB16, and JI18; maize streak resistant lines: CMB5, CMB6, CMB12, CMB13, and CMB14.

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The lines were planted at the Muguga field station, Kenya.

Callus formation and plant regeneration

The procedures for callus induction and plant regeneration conditions were adopted from Bohorova et al. (1999). Immature embryos (1.0-2 mm) of the maize lines were harvested from the field two weeks (12-14 days) after pollination. The ears were cut longitudinally into two segments and surface sterilized by immersion in 70% ethanol for 40 S then 20% chlorox solution, with or without Tween 80 slowly stirring for 20 min. The years were rinsed and sterilized with deionized water 4 times. The embryos were extracted by cutting the top of the kernels with a sharp scalpel blade and placed on prepared N6C1SN media. Harvested ears can be stored for up to 3 days at 4ºC before dissection although the embryos are best isolated immediately. The embryos were placed on the media with the scutellar side up and the embryo axis side in contact with the media. The plates were wrapped with parafilm and foil and incubated at 27ºC in darkness for 3 weeks.

Tissue culture media

The maize callus initiation and maintenance media were based on N6 medium and vitamins (Chu et al., 1975), with added 2.3 g L^{-1} L-proline, 0.2 g L^{-1} casein hydrolysate, 2 ml L^{-1} dicamba, 30 g L^{-1} sucrose, 8 g L^{-1} agar (Bacto) and 0.015 g L^{-1} silver nitrate autoclaved and adjusted to pH 5.7. The maintenance media was the same as for callus initiation minus silver nitrate.

Callus formation and plant regeneration media

The embryogenic calli were transferred to regeneration medium (MSR) containing basal MS medium (Mushirage and Skoog, 1962), 0.040 mg L^{-1} thiamin Cl, 0.15 g L^{-1} L-asparagine, 20 g L^{-1} sucrose, 0.5 ml L^{-1} IAA, 1 ml L^{-1} BAP, 100 mg L^{-1} myo-inositol, 8 g L^{-1} agar autoclaved and adjusted to pH 5.7. The embryonic calli were maintained at 27°C with a 12:12 light/dark photoperiod.

Root formation

The plantlets were transferred to MS medium with 1 mg L^{-1} naphthalene acetic acid (NAA). The plantlets were then transferred into small containers containing peat and kept in a growth chamber covered with a polythene bag for 1 week for adaptation before transferring to the green house.

RESULTS AND DISCUSSION

The development of regenerable tropical maize lines in Kenyan maize breeding programs is crucial to successfully address the many constraints affecting maize production such as drought tolerance, insect pests, diseases and Striga weed menace among others. There has been a concerted effort by many laboratories to develop or identify tropical maize lines suitable for transformation. But the success rate has been low. Temperate lines such as A188 (Ishida et al., 1996) or the hybrid line Hi II (Zhao et al., 1999) have been widely studied and are popularly used for transformation.

We selected 22 maize lines popularly used by the breeders in various ecological zones. The composition was as follows: 5 CIMMYT lines with good combining ability, 7 highland breeder lines, 5 breeder mid altitude lines and 5 breeder lines used for maize streak virus disease resistance development. Our objective in the current work was to use existing protocols to identify maize tropical lines that could be regenerated into plants with ease from tissue cultures for use in transformation projects. The breeder lines were evaluated for their ability to form type II callus using N6C1SN and MSR media (Bohorova et al., 1999) without any modification.

We analyzed the embryogenic response expressed as the percent of embryogenic calli at the eleventh subculture (approx. 5 months after culture initiation) per total number of calli formed 45 d after culture initiation (Tomes and Smith, 1985). We started with an equal number of 25 embryos per plate for each line (Figure 1A). Four of the seven highland lines had a higher embryogenic response; CMB7 (80%), CMB8 (74%), CMB17 (56%) and CMB21 (60%). The same four lines also exhibited ability to form type II callus (Figure 1B). All the lines that developed type II callus proceeded to form many shoots (Figure 1C-D). The small plantlets were separated and subcultured until 4-5 leaves were formed when they were transferred to the rooting media. We did not experience significant loses at the shoot or root induction stage. In our opinion the most critical steps are in obtaining the friable callus in Figure 1B.

With the exception of CIMMYT line Pool A3-6 and msv line CMB5 with embryonic response of 12% and 8% respectively, none of the remaining materials advanced beyond the 45 day subculturing steps for the formation of the type II callus. These calli were non embryogenic, slow growing, hardened and turned dark brown may be due to cell death (Figure 1F). The CIMMYT lines in our collection evaluated here did not form viable callus except for Pool A3-6 showing a comparatively low embryogenic response. However, elite tropical lines CML72, CML216, CML323, and CML327 have been shown to form type I callus (Bohorova et al., 1995). But none of these lines were among the CIMMYT lines collected from breeders in the different ecological zones in Kenya. CMB17 and CMB21 are lines developed from a cross of CMB7 or CMB8 as one of the parents. This observation suggests that genetic background is an important factor in the formation of the type II callus (Fluminhan and Aguiar-Perecin, 1998; Armstrong and Green, 1985). These embryogenic cultures also remained viable over many months of subculturing.

We maintained the media composition and all other growth conditions constant. It was reported by Phillips et al. (1988) that the successful formation of callus depends on several factors among them, the genotype used, and choice of tissue, development stage of the plant, culture media and the environment at each stage of the tissue

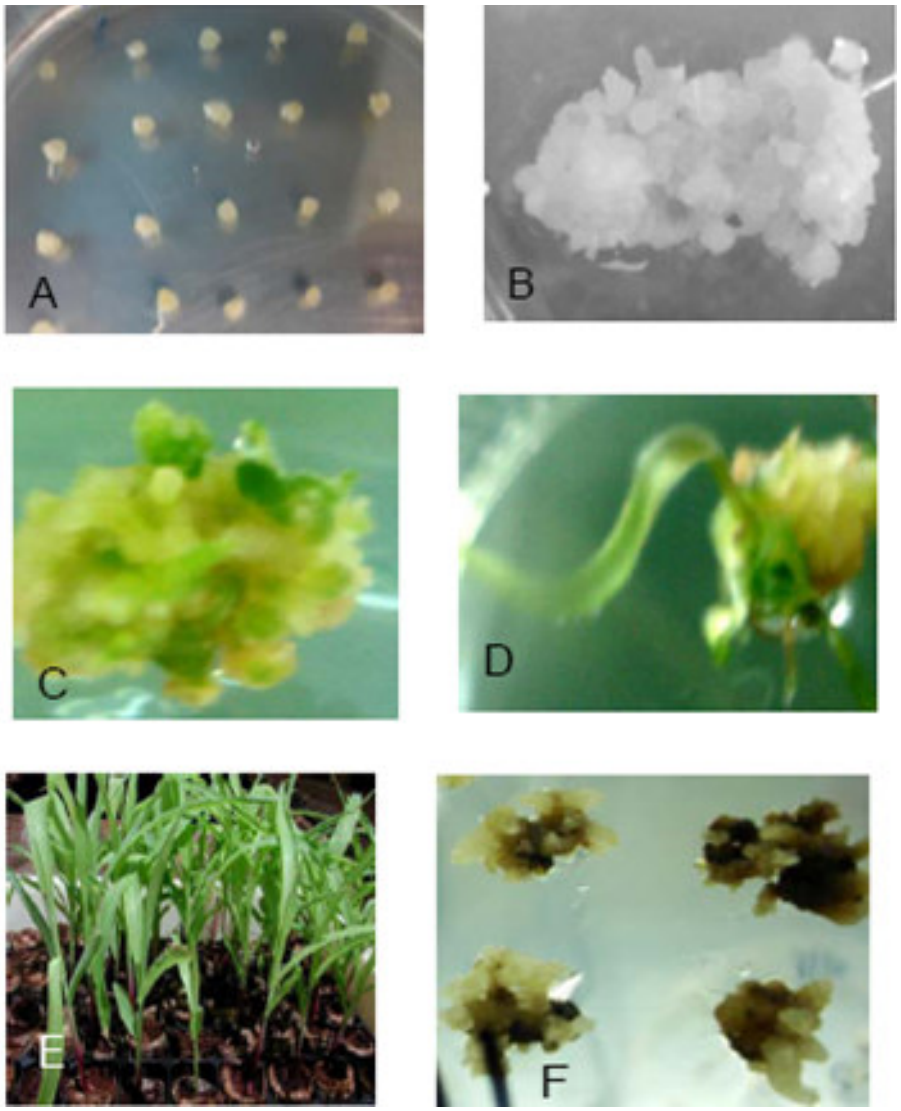

Figure 1. Callus regeneration and conditions: Immature embryos were harvested from twenty two inbred lines**. A**. Approximately 1-2 mm callus were placed on callus induction medium and placed in the dark after wrapping with aluminum foil. The calli were freshly subcultured on the same media every 2-3 weeks by careful selection and splitting of the callus into 3-5 mm pieces before subculturing. **B**. The subculturing of the callus continued for several months until the type II friable well-organized fast growing callus was obtained. **C-D**. After removing the non-embryonic segments, the soft tissue was transferred to the regeneration media (MSR) and exposed to 12:12 light/dark period for shoot induction in baby jars. **E**. After forming 2-3 leaves the plantlets were transferred to root induction media (MSE-NAA). The small plantlets were transplanted in pots containing peat soil. **F**. An example of poor regenerable callus showing calli with hard tissue, retarded growth and dying cells.

culture process. Other major factors are related to age of embryos, placement of embryos on medium and composition of the culture medium (Shohalel et al., 2003; Green and Phillips, 1975). As reported here, our results tend to support previous observations that genotype factors may play a more crucial role in callus formation and plant regeneration (Fluminhan and Aguiar-Perecin, 1998; Lee and Phillips, 1987).

The introduction of suitable foreign genes into the narrow gene pool of lines in use in the Kenyan breeding program is considered important in tackling traits that have proven difficult to address using conventional and molecular marker breeding. Here we report the identification of four lines producing type II callus capable of plant regeneration from immature embryos. The lines which showed good callus formation and plant regenerability are undergoing evaluation for morphological and fertility qualities.

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