Indirect Organogenesis and Multiple Shoots Formation from (*Zea mays* L.) Mature Embryo

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***Abstract*—** Callus induction, regeneration and multiple shoots formation via indirect organogenesis from matured seeds was achieved in five tropical maize genotypes (CML 406, CML 419, CML 424, 425 and CML 427). Explants were cultured on N_6 media supplemented with 5µM 3, 6-dichloro-2-methoxybenzoic acid (Dicamba), 200 mg/l casein hydrolysate and 17.4mM L-Proline. Cultures were maintained in absolute darkness. Calluses were transferred to MS augmented with 8.88 µM Kinetin. Shoots formed were transferred to MS media supplemented with 13.3µM 6-Benzyl amino purine (BAP) for multiple shoots formation. Multiple shoots obtained ranges between 6 and 25 per gram fresh weight of callus. These plantlets were transplanted on rooting media composed of MS augmented with 5µM 1-Naphthaleneacetic acid (NAA). The regenerated plants were acclimatized for two weeks in culture room by translocating them into pots containing equal amount of soil and vermiculite. The acclimatized plants were relocated to glasshouse and grown to maturity. Even though most of the transplanted multiple shoots did not produce ears/seeds in the glasshouse, the study might contribute in better understanding of this recalcitrant and yet important crop.

***Keywords*—** Indirect Organogenesis, Multiple Shoots, Zea mays, Callus, Mature embryo.

I. Introduction

MAIZE or corn as popularly known in many cultures around the globe, is among the most important food crops along with rice and wheat. Fortunately or unfortunately, these leading food crops are all monocots. For many decades, scientist all over the world have been contributing immensely towards the improvement of these important crops particularly maize. Early efforts where solely conventional. However, few decades ago, a more powerful, feasible and dependable approach was devised. This breakthrough was collectively termed as biotechnology-which is a collections of many technologies/techniques such as genetic engineering, cell and tissue culture techniques, molecular cloning and so on and so forth. All these are for the overall enhancement of plant/plant products. However, the success of genetic engineering in plants depends entirely on reliable, efficacious and reproducible tissue culture techniques.

Even though zygotic embryo culture has been in practice for over a century (Raghavan, 2003). Maize embryo culture was first communicated by Green and Philips. Further studies of regeneration from immature embryo-derived maize tissue cultures demonstrated clearly that plant regeneration was from scutellum cells via somatic embryogenesis (Armstrong & Green, 1985), (Lu, Vasil, & Ozias-Akins, 1982). Among the many factors associated with somatic embryogenesis in maize were; developmental stage of the embryo, media composition, type and amount of auxins, additives and most importantly genotype (Armstrong & Green, 1985).

The explant of choice in initiating embryogenic callus lines is undoubtedly immature zygotic embryo. It has been a source of cell suspension cultures (Hansen, 2000), protoplast cultures (Wright et al., 2001) as well as genetic transformation. Interestingly, this is not only applicable in maize but also other monocotyledonous plants such as barley (Chang, Von Zitzewitz, Hayes, & Chen, 2003; Dahleen & Bregitzer, 2002; Kumlehn, Serazetdinova, Hensel, Becker, & Loerz, 2006), millet (Oldach et al., 2001; Rashid, 2002), oat (Gasparis, Bregier, Orczyk, & Nadolska-Orczyk, 2008), rice (Hiei, Ishida, Kasaoka, & Komari, 2006; Hiei & Komari, 2008), sorghum (Hagio, 2002; Nguyen, Thu, Claeys, & Angenon, 2007; Sudhakar, Mani, & Ramana, 2008) and wheat (Wu, Zheng, Liu, & Zhou, 2003). Apart from immature zygotic embryo, immature tassel/inflorescence (Grando et al., 2013; Songstad, Petersen, & Armstrong, 1992) was also successfully employed in plant regeneration via callus stage. Other explants include seedlings (Kotchoni et al., 2012), indirect organogenesis via shoot apex (Muoma, Muluvi, & Machuka, 2008). Plant regeneration through somatic embryogenesis was also achieved using leaves as explant (Ahmadabadi, Ruf, & Bock, 2007). Isolated microspore was also implicated to have formed regenerable callus (Nägeli, Schmid, Stamp, & Büter, 1999).

In general, it is worthy to note that majority of successful work done or protocols that were established, were developed using the maize model genotype A188, B73 and/or their hybrid Hi-II (Che et al., 2006; Songstad et al., 1992) or to a lesser extent H99 (Ishida, Saito, Hiei, & Komari, 2003) and Mo17 (Frame et al., 2006). These genotypes are characterized by high frequency of embryogenic callus proliferation and plant regeneration. These genotypes have poor agronomical value; therefore it would be a time-consuming and costly

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procedure to introduce transgenes into local maize varieties by backcrossing (Lupotto, 1986; Lupotto, Reali, Passera, & Chan, 1999; Lupotto et al., 2004). In a nutshell, the usefulness of immature embryo has been limited by low turn-over, time consuming and expensive because it has to be supplied continuous all year round.

Therefore, the aim of this research was to exploit the qualities of different inbred lines that were to the best of our knowledge not reported before. In addition, to test the competence of mature embryo of these lines in callus induction, maintenance and regeneration.

II. Materials and Methods

A. Plant Materials

Seeds of tropical maize lines were supplied by Centrino Internscional Mejoramiento de Maiz y Trigo (CIMMYT) Mexico. Matured seeds of these lines (CML 406, CML 419, CML 424, 425 and CML 427) were sterilized with 70% ethanol for one minute, rinsed 3-5 times with sterilized distilled water. Thereafter, seeds were treated with commercial bleach (Clorox) containing 50 % (v/v) Sodium Hypochloride with or without 1-2 drops of tween 20 for fifteen minutes and repeated twice. .

B. Callus Induction, Media and auxins optimization

Sterilized seeds were socked in sterilized distilled water for at least 24 hours until the seeds becomes flaccid. Mature embryos were excised with the aid of scalpel blade. Some of the excised embryo was dissected into plumule, radicle and scutellum. The embryonic parts were further re-sterilized in 5% Clorox. With the aid of sterilized pipette tips, they were aseptically rinsed at least three times in sterilized distilled water. Sterilized explants were laid directly on callus initiation on B5 (Gamborg, Miller, & Ojima, 1968), LS, (Linsmaier & Skoog, 1965; Murashige & Skoog, 1962) and N6 (Chu et al., 1975) . These media were augmented with 5 µM of (2, 4-Dichlorophenoxyacetic acid (2, 4-D), 3, 6-dichloro-2-methoxybenzoic acid (Dicamba), Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), 1-Naphthalene acetic acid (NAA), and Picloram). Furthermore, the auxin(s) which gives highest and better callusing response was optimized.

C. Photoperiod Optimization

Cultures were incubated at 27^0C under different photoperiods (24 hours absolute darkness, 24 hours light, 12 hours of light: 12 hours of dark, 16 hours light: 8 hours of dark and 16 hours of dark: 8 hours of light). Callus induced was maintained in same media and same conditions. Primary callus with and without somatic embryos were separated and sub-cultured at regular interval and at different until desired callus lines were obtained

D. Regeneration

Three grams of compact type I callus clumps devoid of somatic embryos were transferred to MS or N6 augmented with benzylaminopurine (BAP) or kinetin (0-25 µM). Regenerated plantlets were transferred to same media with various concentration of cytokinins for multiple shoots formation. Multiple shoots formed were transferred to rooting media constituted with MS or N6 and 5 µM Naphthaleneacetic acid (NAA). Following the establishment of sufficient root system, the plants were transferred to pots containing equal mixture of soil and vermiculite and were acclimatized for 2-3 weeks before being moved to glass house where it was grown to maturity.

E. Data Analysis

In each treatment there was ten explant and replicated three times. ANOVA, Duncan Multiple Range Test for comparison of means at 95% confidence interval and Correlation analysis between and within the groups were extrapolated using IBM SPSS Statistics 21 software

III. Results

A. Callus Induction, Media and auxins Optimization

Callus was initiated in almost all genotypes within six days of inoculation. The envisage scutellum part (middle part of the embryo when plumule and radicle parts are cut off) yielded the highest percentage of callus initiation. The callus formed was loose and yellowish. On the other hand, plumule only gave rise to shoots while majority of radicle part produced roots with very few callus. Of the five auxins screened for optimum callus initiation, Dicamba was observed to have profound effect on both callus ignition as well as callus multiplication (Table. 1). The highest percent (86.67) of primary callus formation was observed in CML 427 in 5 µM Dicamba. While the lowest (3.33) was observed in CML 425 in the presence of Indole-3-acetic acid (IAA). The next best auxin in initiation callus was 2, 4-D. On the other hand, media screening showed that all the genotypes responded very well N6 media for both callus initiation and maintenance. However, MS media was observed to have significantly influences regeneration compared to other media. With respect to photoperiod optimization, it was observed that absolute darkness resulted in highest callus initiation percentage as well as highest growth rate.

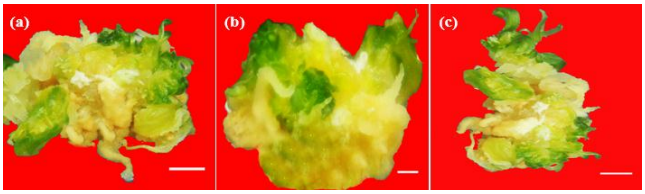

Fig. 1 Organogenic callus lines in regeneration media (a) containing BAP (b) supplemented with kinetin (c) augmented with TDZ. Bar scale = 500 µm (a) and (b) and 250 µm in (c)

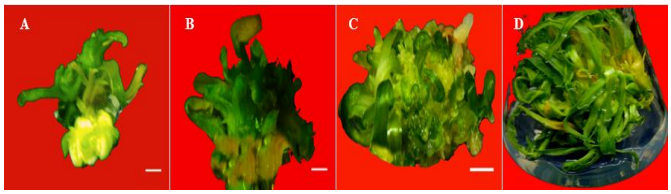

Fig. 2. Multiple shoots formation in media MS media containing 8.8 µM BAP. (A) one week old culture (B) two weeks culture (C) three weeks culture (D) four weeks culture

B. Optimization of Photoperiod

Cultures incubated in absolute darkness showed highest percentage of callus formation. Moreover, the quality and quantity of the callus was significantly higher than the rest. Succeeding 24 hours absolute darkness was 16 hour dark: 8 hours light. Cultures incubated in 24 hours light did not produce any callus, instead, germination was ensued.

TABLE I
UNITS FOR MAGNETIC PROPERTIES

	Genotypes				
PGRs	CML 406	CML 419	CML 424	CML 425	CML 427
2,4-D	61.67^{d}	78.33^{d}	56.67^{c}	58.33^{c}	83.33d
Dicamba	62.22^{d}	80.56^{d}	63.33^{d}	62.22^{d}	86.67^{d}
IAA	6.67^{a}	10.00^{a}	5.00^{a}	3.33^{a}	13.33b
NAA	28.33^{b}	40.00^{b}	31.67^{b}	33.33^{b}	40.00^{b}
Picloram	41.67^{c}	68.33^{c}	53.33^{c}	53.33^{c}	73.33^{c}

Different Letters Differs significantly at $p \leq 0.05$ using Duncan Multiple Range Test

IV. DISCUSSION

Efficient Callus initiation is an essential requirement for any indirect plant regeneration. Culture media also plays a vital role in callus initiation, proliferation and even regeneration. Even though many types of media were formulated and employed in maize tissue culture, the most popular ones are MS and N6. With former being the most widely used. However, other genotypes responds better to N6 media. For example, a friable and embryogenic callus established and maintained using N6 media (Armstrong & Green, 1985). High frequency of plant regeneration from mature embryos via callus stage was also reported using this media (Huang & Wei, 2004). Tissue cultured induced mutation was also studied using maize cultured N6 media (Yu et al., 2011). QTL study on callus induction and totipotency was conducted in maize using N6 media (Krakowsky et al., 2006). Lately, N6 media was also employed as a media choice in other mocots with or without transformation (Bevitori, Popielarska-Konieczna, Dos Santos, Grossi-de-Sá, & Petrofeza, 2013; King, Bray, LaFayette, & Parrott, 2013). In general N6 media have used by many researchers to regenerate the whole plant in maize through callus initiation and maintenance (Al-Abed, Rudrabhatla, Talla, & Goldman, 2006; Danson, Lagat, & Mbogori, 2006; Fu, Li, & Rong, 2005; Manivannan, Kaul, Singode, & Dass, 2010; Rakshit, Rashid, Sekhar, Fatma, & Dass, 2010).

It is a known fact that addition of auxin is essential in callus induction. The type and concentration of auxin required for callusing depends on so many factors which include type of explant, developmental stage of the explant, and genotype among others. In maize tissue culture the most favored auxins are 2, 4-D and Dicamba. In this study it was discovered Dicamba was the preferred auxin having produced highest percentage of callus. Previous studies have suggested that Dicamba was required for the formation of friable type II callus (Carvalho et al., 1997). Regenerable callus tissue from B73 X H99, Pa91, and H99 genotypes were induced and maintained in media supplemented with 3.5 mg/L dicamba (Duncan, Kriz, Paiva, & Widholm, 2003). Friable and embryogenic callus lines were also obtained from tropical maize line (Danson et al., 2006; Rakshit et al., 2010). Bombarded immature embryos were cultured and selected in media containing 3.3 mg/L dicamba (Petrillo et al., 2008). Similarly, dicamba at concentration of 4 mg/L was found to be effective in inducing embryogenic callus in wheat mature embryo (Ren, Wang, & Yin, 2010). Recently, dicamba at 3 mg/L was implicated have promoted genotype independent Somatic embryogenesis from IZE of tropical maize inbred lines (Akoyi et al., 2013).

In contrast to regeneration, multiple shoots were achieved in media augmented with 8.8 µM BAP. Similar trends were reported elsewhere. For example, highest number of multi-shoots developed in medium containing 8.8µM BAP (Li, Masilamany, Kasha, & Pauls, 2002). Similarly, they further observed that Culture media, environmental and genotypic factors are the major issues affecting regeneration from multi-shoot cultures derived from corn seedling apical explants. BAP at 0.5 mg/L was reported to be the optimum concentration for plantlet regeneration and multiple shoot formation through callus initiation from mature maize embryo culture (Huang & Wei, 2004). When callus was formed via split-seed method, regeneration was also reported to be highest at BAP 4.4 µM. Multiple shoots was also recorded at this concentration (Al-Abed et al., 2006). Even though N6 media was found to be the suitable media for callus induction and proliferation but was ineffective when it comes to regeneration. This could be due their differences in nitrogen contents in form of nitrates and ammonium ions or due sulphates ions. In either case, the noticeable differences in both callus initiation and plant regeneration was statistically different. Different types of explants have routinely use induce callus.

V. CONCLUSION

In conclusion, mature embryo could be used as an alternative to immature zygotic embryo especially in basic research. This could circumvent the hurdles involves in procuring IZE. However, the major drawback of this explant is that it is not suitable for establishment of suspension cultures due to the fact that it proportionately yield compact type I callus.

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