ANALYSIS OF MAIZE INBRED LINES FOR THEIR RESPONSE TO SOMATIC EMBRYOGENESIS

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Abstract: Screening of five maize inbred lines for their ability to form somatic embryos done. The immature embryos were used as explants for callus induction. Embryogenic calli were induced on MS supplemented with ten different concentrations of growth hormones, 2, 4-Dichlorophenoxyacetic acid, 1-naphthalene acetic acid, 6-benzylamino purine, kinetin, picloram and sucrose solidified with agar. LM 13 showed highest callus induction and subsequent embryogenesis, although the results were statistically non-significant. The somatic embryos obtained were further subcultured on to regeneration medium for studying the regeneration potential of the inbreds.

Key words: Somatic embryos, Immature embryos, embryogenesis, Inbred lines

INTRODUCTION

Maize is a domesticated cereal grain that has been grown as food and animal feed for thousands of years. At present, it is the most widely grown crop in the world, and is now at the foundation of a new agricultural revolution, where the grains are used as factories to manufacture of high-value products from maize [1]. Maize seeds are excellent production vehicles because of their large size compared to other cereals, with 82% of the seed made up of endosperm [2]. In tropical farming systems, the gap between the actual yields and achievable is quite large because of various abiotic (aluminium toxicity, excessive moisture, acid soil, drought, low nitrogen) and biotic (diseases, insect-pest) stresses in particular ecological and geographic environments [3-5]. Even the finest genetic materials often do not acquire the tolerance and resistance needed to overcome the stresses encoured. Traditional breeding has restricted success to overcome these constraints because of non-availability of genetic resistance in germplasm, high irregularity and complexity of abiotic stress traits.

However, use of molecular breeding and genetic transformation hold great potential to overcome these constraints. A reliable in vitro regeneration of normal and fertile plant from single cell, tissue and organ is the basic prerequisite for the production of genetically modified plants. Regeneration from embryogenic type II calli derived from immature embryos has been the most reproducible transformation/regeneration system described for maize [6-10]. The achievement of different biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation strongly depends on an efficient recovery of plants through in vitro systems. Somatic embryogenesis is the most common technique of in vitro plant regeneration [11]. In maize compact embryogenic calli arises at low frequency. Embryogenesis and subsequent plant regeneration capability has been reported to be influenced mainly by genotype and media compositions [12,13] in addition to other factors. The present studies were performed to
identify the inbred lines that respond well to embryogenic callus induction and plant regeneration from immature embryos so that they can be utilized for genetic transformation.

MATERIALS AND METHODS

Five inbred lines (LM 5, LM 6, LM 13, LM 15 and LM 16) were obtained from Maize section, Department of Plant Breeding and Genetics, Punjab Agricultural University Ludhiana. These inbred lines were identified as parents of foremost maize hybrids in commercial production. The surface sterilization of immature grains was done using mercuric chloride (0.1%) for 10 minutes. The treatment was followed by three washing with autoclaved distilled water. The excised immature embryos (with scutellar side exposed and embryo axis in contact with medium) were cultured on the callus induction medium composed of MS salts [14] containing various concentrations and combinations of auxins (picloram, 2,4-D, NAA) with or without cytokinins (BAP, Kn) in addition to sucrose (60g/l) solidified with agar (8g/l). Different growth regulator combinations evaluated were:

a) 2, 4-D (3.0, 6.0, 10.0 mg/l) + Kn (0.75 mg/l)
b) 2, 4-D (3.0 mg/l) + Picloram (5, 10 mg/l)
c) NAA (5,10 mg/l) + Kn (0.75 mg/l)
d) Picloram (2.5, 5, 10 mg/l) + BAP (0.5 mg/l)

Selection of best medium for all the five inbreds were tested for their response. The callus induced was then sub-cultured on maintenance medium with low concentrations of auxins [MS containing 2, 4-D (1.5, 3.0, 5.0 mg/l), NAA (2.5, 5, mg/l), picloram (2.5, 5, mg/l) and sucrose (30g/l)]. All the chemicals used were of analytical grade (Hi Media, India). Cultures were incubated at 25 ± 2°C in the dark. The experiment was repeated three times in completely randomized block design with thirty explants per replication. Different inbred lines were compared using LSD.

RESULTS AND DISCUSSION

To optimize the cultural conditions for callus induction in maize, using immature embryos as explants, ten media compositions based on MS salts and supplemented with different combinations and concentrations of auxins and cytokinins were investigated (Table 1, Graph 1). The cultured embryos with their embryo axis in contact with the medium exhibited swelling after 10-12 days of incubation. Culturing the embryos with embryo axis side down, inhibited root elongation and facilitated callus initiation (Fig. 1,2).

The auxins play important role in cell elongation, whereas, cytokinins are essential for cell division. Initial swelling of explants on callusing medium was the result of rapid cell division caused by cytokinins and cell elongation caused by auxins, picloram and 2,4-D. Picloram is considered similar in action but potent than 2,4-D. Several studies have established that picloram exhibits growth-regulating properties of the auxins [15-17]. In the present studies, callus initiation from immature embryos was observed after fifth day of culturing on callus induction medium from the surface of scutellum (Fig. 2) which is in coherent with the findings of Huang and Wei [18] and Abebe [19]. This may be attributed to the presence of meristematic cells in the scutellum. Al-Abed have reported the presence of the meristematic cells in the scutellum of maize embryos which proliferate into callus [20]. Certain problems associated with the use of 2,4-D in tissue culture were not encountered when picloram was substituted for 2,4-D in the culture medium of 2,4-D requiring plant material, is well documented [21]. Picloram performed as well or better than the auxins to which it was compared when used in plant tissue cultures. In the present study, the percentage of the primary callus formed ranged from 40.3% to 88.7% for immature embryos (Table 1, Graph 1). Frequency of callus induction was highest (88.7%) on MS medium supplemented with Picloram (10.0 mg L⁻¹) + 2,4-D (3.0 mgL⁻¹) + sucrose (3%) solidified with agar (8 gL⁻¹). Picloram has also been used as a auxin substitute in callus cultures [22,23]. Depending on different types of media used embryogenic callus was first formed after the first subculture on callus maintenance medium. Globular presomatic embryos were formed on the surface of the embryogenic callus (Fig. 3). Two types of embryogenic calli were formed, type I and type II callus. Type I callus was compact, white to cream, while type II callus was friable and light yellow. The formation of type I and type II calli has been reported in maize [24,25]. Genotype specificity of somatic embryogenesis and regeneration in maize is well documented [24-27]. Variation of the genotypes to form type I and type II callus in maize has been reported to be due to additive gene effects, while heterosis has been found to increase cultural response.
Table 1: Per cent callus induction from immature embryos of five maize inbreds on different media compositions

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Medium Code</th>
<th>LM 5</th>
<th>LM 6</th>
<th>LM13</th>
<th>LM15</th>
<th>LM16</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 2,4-D (3.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M1</td>
<td>44.3±0.8 (41.7)</td>
<td>47.7±1.4</td>
<td>40.3±1.4 (39.4)</td>
<td>45.7±1.4 (42.5)</td>
<td>46.3±0.8 (42.9)</td>
</tr>
<tr>
<td>MS + 2,4-D (6.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M2</td>
<td>44.7±0.8 (41.9)</td>
<td>50.3±1.7 (45.2)</td>
<td>52.3±2.0 (46.3)</td>
<td>43.7±0.8 (41.8)</td>
<td></td>
</tr>
<tr>
<td>MS + 2,4-D (10.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M3</td>
<td>46.7±1.2 (43.1)</td>
<td>40.7±3.1 (39.6)</td>
<td>52.3±2.0 (46.3)</td>
<td>43.7±0.8 (41.8)</td>
<td></td>
</tr>
<tr>
<td>MS + NAA (5.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M4</td>
<td>45.3±1.4 (42.3)</td>
<td>49.7±1.7 (44.8)</td>
<td>41.7±1.2 (40.2)</td>
<td>47.7±3.2 (43.6)</td>
<td></td>
</tr>
<tr>
<td>MS + NAA (10.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M5</td>
<td>51.7±2.1 (45.9)</td>
<td>44.3±0.8 (41.7)</td>
<td>43.7±0.8 (41.3)</td>
<td>41.3±2.0 (40.0)</td>
<td></td>
</tr>
<tr>
<td>MS + 2,4-D (3.0) mgL⁻¹ + Pic (5.0) mgL⁻¹</td>
<td>M6</td>
<td>78.3±0.8 (62.3)</td>
<td>76.3±1.4 (60.9)</td>
<td>72.7±0.8 (58.5)</td>
<td>75.3±2.0 (60.2)</td>
<td></td>
</tr>
<tr>
<td>MS + 2,4-D (3.0) mgL⁻¹ + Pic (10.0) mgL⁻¹</td>
<td>M7</td>
<td>78.3±1.4 (62.3)</td>
<td>80.3±0.8 (63.7)</td>
<td>79.6±1.7 (63.2)</td>
<td>80.6±1.4 (63.9)</td>
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<tr>
<td>MS + NAA (2.5) mgL⁻¹ + BAP (0.5) mgL⁻¹</td>
<td>M8</td>
<td>47.6±1.7 (43.6)</td>
<td>49.3±1.2 (44.6)</td>
<td>43.3±1.7 (41.1)</td>
<td>46.3±0.8 (42.9)</td>
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</tr>
<tr>
<td>MS + NAA (5.0) mgL⁻¹ + BAP (0.5) mgL⁻¹</td>
<td>M9</td>
<td>58.7±2.0 (49.9)</td>
<td>62.7±0.8 (52.3)</td>
<td>53.3±1.4 (46.9)</td>
<td>58.3±0.8 (49.8)</td>
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<tr>
<td>MS + Pic (10.0) mgL⁻¹ + BAP (0.5) mgL⁻¹</td>
<td>M10</td>
<td>62.7±1.2 (52.3)</td>
<td>70.3±2.0 (57.0)</td>
<td>62.7±1.4 (52.3)</td>
<td>57.3±0.8 (49.2)</td>
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Table 2: Percent somatic embryogenesis induction in immature embryos of five maize inbreds on different media compositions

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Medium Code</th>
<th>LM 5</th>
<th>LM 6</th>
<th>LM13</th>
<th>LM15</th>
<th>LM16</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS + 2,4-D (1.5) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M11</td>
<td>31.3±2.6 (34.0)</td>
<td>28.0±2.8 (31.7)</td>
<td>31.3±2.3 (34.0)</td>
<td>27.6±2.3 (31.7)</td>
<td></td>
</tr>
<tr>
<td>MS + 2,4-D (3.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M12</td>
<td>39.6±4.4 (39.0)</td>
<td>32.3±2.6 (34.6)</td>
<td>46.3±2.1 (42.9)</td>
<td>30.6±3.3 (35.5)</td>
<td>30.3±2.4 (33.4)</td>
</tr>
<tr>
<td>MS + 2,4-D (5.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M13</td>
<td>28.3±2.3 (32.1)</td>
<td>21.0±2.5 (27.2)</td>
<td>20.6±2.6 (26.7)</td>
<td>24.6±1.7 (29.7)</td>
<td>21.3±2.6 (27.4)</td>
</tr>
<tr>
<td>MS + NAA (2.5) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M14</td>
<td>22.6±2.0 (28.4)</td>
<td>23.3±2.9 (28.8)</td>
<td>29.3±3.5 (32.7)</td>
<td>23.6±2.6 (29.0)</td>
<td>25.3±2.9 (30.1)</td>
</tr>
<tr>
<td>MS + NAA (5.0) mgL⁻¹ + Kn (0.75) mgL⁻¹</td>
<td>M15</td>
<td>27.0±3.2 (31.2)</td>
<td>20.6±2.0 (27.0)</td>
<td>23.3±2.3 (28.8)</td>
<td>22.3±2.4 (28.1)</td>
<td>19.0±2.3 (25.8)</td>
</tr>
<tr>
<td>MS + 2,4-D (1.5) mgL⁻¹ + Pic (1.0) mgL⁻¹</td>
<td>M16</td>
<td>30.3±3.4 (33.3)</td>
<td>28.3±2.0 (32.1)</td>
<td>39.6±1.4 (39.0)</td>
<td>32.0±2.3 (34.4)</td>
<td>29.0±2.6 (32.5)</td>
</tr>
<tr>
<td>MS + 2,4-D (3.0) mgL⁻¹ + Pic (2.5) mgL⁻¹</td>
<td>M17</td>
<td>70.6±4.3 (57.3)</td>
<td>63.0±2.6 (52.5)</td>
<td>75.3±2.9 (60.3)</td>
<td>56.3±2.9 (48.6)</td>
<td>35.3±3.3 (36.4)</td>
</tr>
<tr>
<td>MS + Pic (1.0) mgL⁻¹ + BAP (3.0) mgL⁻¹</td>
<td>M18</td>
<td>25.3±2.7 (30.1)</td>
<td>23.6±2.9 (29.9)</td>
<td>21.3±2.9 (27.4)</td>
<td>18.3±3.4 (25.1)</td>
<td>19.6±2.1 (26.3)</td>
</tr>
<tr>
<td>MS + Pic (2.5) mgL⁻¹ + BAP (3.0) mgL⁻¹</td>
<td>M19</td>
<td>28.0±2.7 (32.3)</td>
<td>25.6±3.2 (30.3)</td>
<td>24.6±3.2 (29.7)</td>
<td>19.6±1.7 (26.3)</td>
<td>27.3±3.1 (31.4)</td>
</tr>
<tr>
<td>MS + Pic (5.0) mgL⁻¹ + BAP (3.0) mgL⁻¹</td>
<td>M20</td>
<td>34.3±4.2 (35.8)</td>
<td>37.3±2.0 (37.6)</td>
<td>61.0±2.0 (51.3)</td>
<td>32.0±3.2 (34.4)</td>
<td>29.6±2.3 (33.0)</td>
</tr>
</tbody>
</table>

positively [24]. In conformity with the earlier reports [28] the type II callus arised at low frequency. The two types of calli were also observed on the same embryo [29]. It is well documented that juvenile tissues are usually more responsive to tissue culture than mature ones [18,30]. Non embryogenic callus was also formed which was soft, watery and yellow in colour after 2 weeks of incubation (Fig. 4). The formation of embryogenic and non-embryogenic callus from immature embryo has been reported in several studies [31]. Successful establishment of competent cells which can regenerate into whole plants, largely depends on the capability of identifying the appropriate cell type. In this study, swelling of the scutellar tissue of immature embryo cultured on a medium with 2,4-D was observed. Studies have shown that 2,4-D has been the important factor in the initiation and proliferation of primary and embryogenic calli from immature embryos of maize [28,32], mature embryos of maize [18] and from Dhillon and Gosal.
Graph 1: Per cent callus induction from immature embryos of five maize inbreds on different media compositions

Graph 2: Percent somatic embryogenesis induction in immature embryos of five maize inbreds on different media compositions.

mature and immature embryos of wheat [33]. In the present study high concentration of 2,4-D reduced callusing. This could be due to the blockage of cell division and inactivation of cells which have the embryogenic potential.

Concentration of 2,4-D had a significant effect on the percentage of the primary and embryogenic calli initiated from immature embryos. The statistical comparison of ten different media compositions used for callus induction from immature embryos of five inbred lines (LM5, LM6, LM13, LM15 and LM16) revealed that best media compositions, i.e., MS + 2,4 D (3 mgL⁻¹) + picloram (10.0 mgL⁻¹) was significantly better than rest of the nine media compositions for all inbred lines. Picloram has been used to induce callusing even in recalcitrant genotypes [17]. There were genotypic differences with respect to callus induction. For LM5, the callus induction on two media, i.e., MS + 2,4 D (3 mgL⁻¹) + picloram (10.0 mgL⁻¹) and MS + 2,4 D (3 mgL⁻¹) + picloram (5.0 mgL⁻¹) concentrations were statistically at par. For inbred LM6, media MS + 2,4 D (3 mgL⁻¹) was found to be the best and callus induction was significantly better than other nine media. Among different inbreds, LM13 showed highest number of calli that was significantly better than the number of calli induced in the other inbreds on the same medium. Therefore, MS + 2,4 D (3 mgL⁻¹) + picloram (10.0 mgL⁻¹) was found to be the best medium for
Fig. 1: Callus induction from cultured immature embryos of LM13 cultured on MS+ 2,4-D (3.0) mgL\(^{-1}\) + Pic (10.0) mgL\(^{-1}\) after 12 days of incubation.

Fig. 2: Culturing the immature embryos of LM6 with embryo axis side down, exhibiting callus induction after 10-12 days of incubation on MS + 2,4-D (3.0) mgL\(^{-1}\) + Pic (5.0) mgL\(^{-1}\).

Fig. 3: Showing the globular somatic embryos from callus of LM13 immature embryos on MS+ Pic (5.0) mgL\(^{-1}\) + BAP (0.5) mgL\(^{-1}\).

Fig. 4: Soft, watery and yellowish non embryogenic callus developed from immature embryos cultured on MS + 2,4-D (3.0) mgL\(^{-1}\) + Pic (10.0) mgL\(^{-1}\) after 2 weeks of incubation.

Fig. 5: Showing whitish embryogenic calli on yellowish non embryogenic calli developed from LM13 inbred cultured on reduced level of auxin after 5-6 weeks.
embryogenic callus induction in maize inbred.

**Maintenance and multiplication of calli:** The developed calli were maintained and multiplied by excising primary calli after 5-6 weeks for immature embryos driven calli (Fig. 5) and subsequent culturing on proliferation medium with reduced levels of auxins, such as, picloram and 2,4-D. The subculturing was done after every two weeks. Callus was maintained by subculturing after every 3-4 weeks. Philippe had maintained and multiplied callus cultures through periodic subculturing on MS medium containing AgNO3 after every two weeks [34]. Similarly, Carvalho maintained and multiplied calli on MS medium containing AgNO3 after every two weeks [28]. Agar nutrient medium containing 2,4-D (0.5 mgL⁻¹) and sucrose (2%) has been reported as most conducive for embryogenic calli from immature embryos of maize [35]. Similarly, N₆ medium supplemented with 2,4-D (2.0 mgL⁻¹) + AgNO₃ (10 mgL⁻¹) + proline (300 mgL⁻¹) + casein hydrolysate (500 mgL⁻¹) and sucrose (20 gL⁻¹) was found to be the best for shoot generation from embryogenic calli initiated from immature embryos of maize [36]. Frequent somatic embryogenesis has been reported in immature embryo derived maize calli on N₆ medium supplemented with proline, casein hydrolysate, silver nitrate and sucrose (2%) using different concentrations of 2,4-D [11]. Immature embryos has been proven to be the best source for the establishment of embryogenic callus and plant regeneration in maize [12,24,25,37-39]. Further, the initiation and maintenance of tissue culture in maize using immature embryos depends mainly on the age of embryos, placement (orientation) of embryos on medium and composition of culture medium[37].

**Induction of somatic embryogenesis:** Somatic embryogenesis plays an important role in cloning and genetic transformation of plants. Indirect somatic embryogenesis, i.e., development of somatic embryos from callus tissue, is a multistep procedure which requires a lengthy time interval for the development of whole plants [40]. Whereas, direct somatic embryogenesis is advantageous over indirect somatic embryogenesis as a shorter period is needed for obtaining somatic embryos [41], the reduction in the culture period also decrease the frequency of somaclonal variation [42]. In the current investigation, different media compositions were tried for induction of somatic embryogenesis (Table 2, Graph 2). Somatic embryogenesis from immature embryos was observed after 20-25 days of culturing (Fig. 5). The embryogenic calli obtained was further subcultured on to regeneration medium for studying the regeneration potential of the inbreds.

This effort sets as a starting point for developing more efficient protocol for in vitro plant regeneration and their further use in genetically transforming the inbred independently of the gene transfer method chosen to compliment conventional breeding efforts.

**Abbreviations used:** MS: Murashige and Skoog medium, 2,4-D: 2, 4-Dichlorophenoxyacetic acid, NAA: 1-naphthalene acetic acid, BAP: 6-benzylamino purine, Kn: Kinetin

**REFERENCES**