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# *A Study on the Integration of In Vitro Methods with In Vivo Double Haploid Technique in Maize (Zea mays L.)*

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***ABSTRACT:*** *The in vivo doubled haploid technique in maize breeding significantly reduces the time required for developing homozygous lines, offering advantages in terms of both time and cost. Although this technique enables the development of lines much faster than traditional breeding methods, ongoing research aims to further shorten the development process through alternative approaches. In this context, significant efforts have been devoted to integrating in vitro methods with in vivo doubled haploid technique. This study aimed to investigate the potential of combining in vivo and in vitro techniques for the rapid development of homozygous maize lines. A total of 10 local populations and 3 inducer lines (CIM2GTAIL-P2, ADAIL-1, STOCK-6) were used as experimental material. The study was conducted in two phases under field and laboratory conditions. During the first phase, induction crosses were performed in 2022, and the haploid induction rates of donor genotypes were found to range from 1.29% to 7.12%, as determined using the Navajo marker. In the laboratory phase, immature embryo culture was employed for both direct and indirect regeneration using samples collected 18–20 days after induction crossing. Haploid status of the samples obtained through direct regeneration was confirmed using the Feulgen chromosome staining method. Four of the donor materials (DON3, DON4, DON6, DON7) yielded successful results in tissue culture studies. Explants were taken from immature embryos to CHU medium for callus formation and then these calli were transferred to Murashige and Skoog medium for the formation of somatic embryos. This approach enabled the production of 3 to 6 calluses per immature embryo, depending on the donor genotype. The results of this study indicate that integrating immature embryo culture as an in vitro method into the in vivo doubled haploid technique can offer benefits in terms of both time efficiency and an increased number of developed materials.*

***Keywords: Zea mays L.****, callus, haploid induction rate, embryo, homozygous, tissue culture.*

## *Mısırda (Zea mays L.) In vivo Katlanmış Haploid Tekniğine In vitro Yöntemlerin Entegrasyonu Üzerine Bir Çalışma*

***ÖZ:*** *Mısır ıslahında kullanılan in vivo katlanmış haploid yöntemi, homozigot hatların geliştirilme süresini önemli ölçüde kısaltarak ıslah programlarına hem zaman hem de maliyet açısından avantaj sağlamaktadır. Klasik yöntemlere kıyasla daha kısa sürede hat elde edilmesine olanak tanıyan bu yöntem, geliştirme sürecinin daha da hızlandırılabilmesi için farklı stratejilerle desteklenmektedir. Bu bağlamda, in vitro yöntemlerin in vivo haploid tekniklerine entegre edilmesi üzerine yoğun bir araştırma yürütülmektedir. Bu çalışmada, in vivo ve in vitro yöntemlerin bir arada uygulanmasıyla kısa sürede homozigot mısır hatlarının geliştirilme potansiyeli incelenmiştir. Araştırmada, 10 yerel popülasyon ve 3 indirgeyici hat (CIM2GTAIL-P2, ADAIL-1, STOCK-6) donör materyal olarak kullanılmıştır. Denemeler, tarla ve laboratuvar koşullarında iki aşamalı olarak gerçekleştirilmiştir. İlk aşamada, 2022 yılında indüksiyon melezlemeleri yapılmış ve Navajo markörüne göre değerlendirilen donör hatların indirgenme oranları %1,29 ile %7,12 arasında değişmiştir. Laboratuvar aşamasında, indüksiyon melezlemesinden 18-20 gün sonra alınan örnekler kullanılarak olgunlaşmamış embriyo kültürü yöntemiyle hem doğrudan hem de dolaylı rejenerasyon sağlanmıştır. Doğrudan rejenerasyon yoluyla elde edilen örneklerin gerçek haploid oldukları, Feulgen kromozom boyama yöntemiyle doğrulanmıştır. Çalışmada kullanılan donör materyallerden dört tanesi (DON3, DON4, DON6, DON7), doku kültürü aşamalarında başarılı sonuçlar vermiştir. Olgunlaşmamış embriyolardan kallus oluşumu için CHU besi ortamına eksplantlar alınmış sonrasında bu kallıuslardan somatik embriyoların oluşması için eksplantlar MS ortamına transfer edilmiştir. elde edilerek somatik embriyolar oluşturulmuş ve bu embriyolar alt kültürlere aktarılmıştır. Bu yöntemle, bir embriyodan tür özelliklerine bağlı olarak 3 ila 6 arasında klon bitki üretilebilmiştir. Araştırma sonuçları, in vivo haploid tekniğine olgunlaşmamış embriyo kültürünün entegre edilmesinin zaman tasarrufu sağladığını ve geliştirilen materyal miktarını artırdığını ortaya koymuştur.*

***Anahtar kelimeler: Zea mays L.,*** *kallus, haploid indirgeme oranı, embriyo, homozigot, doku kültürü.*

## INTRODUCTION

Maize, whose homeland is America, is a warm climate plant with high adaptability and is cultivated in different parts of the World. The USA and China produce the most maize worldwide (FAO, 2024). In Türkiye, maize is mostly used as a raw material in human nutrition, animal feed and as starch in industry. According to the data of Turkish Statistical Institute (TURKSTAT, 2022), despite a decrease in cultivation area in 2022, increases in yield and production were observed. Obtaining new varieties is an important parameter in achieving high yields in maize (Dwivedi *et al*., 2015). In Türkiye, maize was grown on 800.000 hectares with 6.9 million tons of grain production realized (FAO, 2024). The proportion of foreign varieties in hybrid maize seeds is approximately 95% in Türkiye, while the proportion of local varieties does not exceed 5% significant amounts of royalty fees are paid abroad every year for foreign varieties. This situation makes the development of national varieties strategic. Maize varieties are classified in seven main groups according to their endosperm structure. These are dent maize, flint maize, popcorn maize, sweet maize, flour maize and waxy maize (Kahrıman *et al*., 2022a). The most used varieties are dent maize and flint maize. Popcorn and sweet maize are used by people as snacks. The use of maize as a raw material in the food, textile, chemical and pharmaceutical industries cause the demand for maize to expand with the increase in the World population. To meet this demand, development of high yielding and high-quality maize varieties is required (Kahrıman *et al.*, 2013).

The methods used with maize differ in terms of breeding, production and genetic aspects. The *in vivo* haploid technique in maize is used to make homozygous technologies more widespread. The classification of haploid and diploid is the most important stage of this technique (Prasanna *et al*. 2012). With the *in vivo* haploid method, homozygous lines can be developed in a very short time. There are two methods for haploidization: paternal (androgenetic) and maternal (gynogenetic) haploid methods. In the maternal haploid (gynogenetic) method, haploid embryos are obtained from lines pollinated with inducer genotypes (Dwivedi *et al*., 2015; Yorgancılar *et al*., 2019). Pollen from the inducer line triggers the egg cell to develop an embryo containing only haploid maternal genome. Thus, inducers used in the maternal haploid technique can produce seeds with haploid embryos when used to pollinate other genotypes. In the paternal haploid (androgenetic) method, the inducer line is used as the pollen receiver. The cytoplasm of paternal haploids is formed from the inducer line, but the chromosomes belong only to the donor plant. The haploid induction rate resulting from the maternal haploid method is much higher compared to the paternal haploid technique (Lashermes and Beckert, 1988). Therefore, in practice, the maternal haploid technique is preferred to the paternal haploid technique in maize breeding programs. The production of homozygous lines by the *in vivo* doubled haploid technique shortens the development process and consequently increases the genetic gain. Because of these advantages, this technique has been widely used in maize breeding programs.

Accelerated breeding approaches integrated into the *in vivo* doubled haploid technique can provide significant advantages. Traditional *in vivo* doubled haploid (DH) technology can significantly increase the efficiency of breeding programs by producing homozygous lines in only two generations. By integrating accelerated breeding methods into this technique, it is possible to shorten the breeding process even more. In this way, immature embryos obtained from donor materials by induction hybridization (60-80 days) can be cultured *in vitro* (20-30 days) and homozygous lines can be obtained after acclimatization to the field. Thus, with the *in vivo* doubled haploid technique (without the use of greenhouse), it may be possible to reduce the studies that take 18-20 months to 5-6 months. This study was aimed i) to evaluate the haploid induction rate of different donor materials crossed with three inducer lines, and ii) to investigate the possibility of integration of immature embryo culture practices into the *in vivo* doubled haploid technique.

## MATERIAL AND METHOD

### Experimental material

In the study, 10 donor materials (HYAxB73, ORDU ULUBEY, POP1, TR37799, POP2, BAP81, TR37105, TR38823, TR55533, IND) and 3 inducer lines (Stock-6, ADAIL-1, CIMP2GTAIL-P2) were used as experimental materials.

**Establishment of field trial and induction hybridization**

The field trial was established at Çanakkale Onsekiz Mart University, Faculty of Agriculture, Crop Production Research and Application Unit in 2022. Donor materials were sown in 4 row plots and inducer lines were sown in 15 row plots. Sowing density was 70 cm x 20 cm. Drip irrigation was used in the experiment field and the first irrigation was carried out immediately after sowing. Fertilization was applied with the irrigation system according to (Anonim, 2022). Hand pollination was applied, and the ears were protected in order to prevent cross pollination. When the donor materials and inducer lines reached flowering, at least 8-10 cobs from each donor were pollinated with pollen collected from inducer lines Approximately 18-20 days after pollination, the cobs were harvested and the seeds which were examined by R1-nj marker and accepted as haploid were taken into tissue culture studies.

The ears were collected from the plants that reached harvest maturity in such a way that they would not be mixed and were placed in separate bags. The samples were manually shelled and the seeds from each sample were kept under appropriate conditions until classification was carried out. In this classification, haploid, diploid and hybrid seeds were determined by Navajo marker as proposed by (Prasanna *et al*., 2012). Based on these numbers, HIR values were determined with the following formula.

Haploid Reduction Rate (HIR-%) = Number of Haploid Seeds/ Total Number of Seeds

**Immature embryo culture**

In this study, the integration of immature embryo culture into the *in vivo* doubled haploid technique was tested with two different approaches. In the first approach, a plant was obtained from a single immature embryo or seed by direct regeneration and clonal propagation of this plant by callus culture was examined. In the second approach, immature embryos were first tested in callus culture and then somatic embryos formed from these calli were subjected to chromosome doubling, subcultured and their development was monitored. For the sterilization of seed samples to be used in both direct and indirect methods, seeds were first subjected to surface sterilization. In this context, the samples were kept in 70% ethanol for 30 s and then passed through pure water and kept in 5% sodium hypochlorite for five seconds before the extraction of embryos. Immature embryos extracted from these samples were not subjected to any additional sterilization and were cultured according to the methods described below (Figure 1).

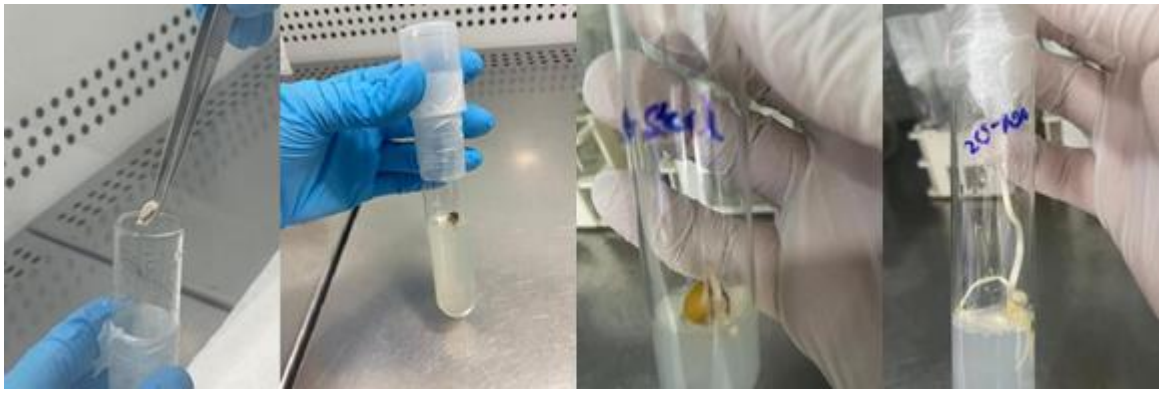

Figure 1. Culture of matured embryo and early seed in preliminary studies.

**Direct Regeneration:** This method was used to regenerate immature embryos on Murashige and Skoog (1962) medium. In the experiment, MS medium that contained (1650 (mg/L) $NH_4NO_3$, 1900 (mg/L) $KNO_3$, 440 (mg/L) CaCl22H2O, 370 (mg/L) $MgSO_4$.7H2O, 170 (mg/L) $KH_2PO_4$, 0.83 (mg/L) Kl, 6.6 (mg/L) $H_3BO_3$, 22.3 (mg/L) $MnSO_4$.4H2O, 8.6(mg/L) $ZnSO_4$.7H2O ,0.03 (mg/L) $CuSO_45H_2O$, 0.03 (mg/L) $COCl_2$.6H2O, 27.8 (mg/L) $FeSO_4$.7H2O, 37.3 (mg/L) $Na_2EDTA.2H_2O$, 100 (mg/L) Inositol ,0.5 (mg/L) Nicotinic acid, 0.5 (mg/L) Pyridoxine-HCl, 0.1 (mg/L) Thiamine-HCl,, 2 (mg/L) Glycine, and 3% Sucrose) was used to obtain plants from immature embryo/seed samples.

**Indirect Regeneration:** This method was based on the technique proposed by Chu *et al*. (1975), in which immature embryos are first placed in CHU medium for embryonic callus formation, and then these embryonic calluses are subcultured in MS medium to obtain *in vitro* explants. In this methods , CHU medium (463.000 (mg/L) $NH_42SO_4$, 125.33 (mg/L) $CaCl_2$, 2830.0 (mg/L) $KNO_3$, 90.370 (mg/L) $MgSO_4$ , 1600 (mg/L) $KNO_3$, 400.0 (mg/L) $KH_2PO_4$ 1.6(mg/L) $H_3BO_3$, 37.300 (mg/L) $C_{10}H_{14}N_2O_82Na.2H_2O$, 27.800 (mg/L) $FeSO_47H_2O$, 3.300 (mg/L) $MnSO_4.H_2O$, 0.800 (mg/L)KI, 1.50 (mg/L) $ZnSO_4.7H_2O$, 0.500 (mg/L) Nicotinic Acid, 0.500 (mg/L) Pyridoxine HCL, 2.000 (mg/L) Glycine, and 20000.0 (mg/L) Sucrose) were used.

**Immature embryo procedures**

Haploid maize grains from the ear samples obtained from the field trial were separated from the seed with the help of a scalpel so as not to damage the embryos. For the sterilization of seed samples to be used in both direct and indirect methods, they were first subjected to surface sterilization. In this context, the samples were kept in 70% ethanol for 30 s and then passed through pure water. Immature embryos extracted from these samples were not subjected to any additional sterilization and were cultured in the MS and CHU media according to method used. Culture media was prepared in the laboratory in a sterile cabinet in accordance with the proposed by Murashige and Skoog (1962) and Chu *et al*. (1975). In this context, inorganic compounds, organic compounds, vitamins, hormones, growth regulators were weighed and mixed. Contaminacide (0, 3 and 6 ml/L) were used for maintaining tissue culture aseptic conditions as suggested by Türkmen *et al.* (2023). Then pH was adjusted to 5.8 and distilled water was added to 1 liter. Lastly agar (8 g/l) was added to solidify media.

The MS or CHU medium was transferred to sterile tubes/boxes before autoclaving and protected against contamination by wrapping with parafilm tape. The samples were placed in the autoclave in order to ensure sterilization. The appropriate sterilization program for the samples was set at 121 °C for 20 minutes. After sterilization, the explants were cultured in the sterile cabin in accordance with the prepared medium without damaging the embryo part of the seed with the help of forceps, scalpel, petri dish, bunsen burner.

To provide suitable light, temperature and humidity conditions for the culturing of plant tissues, the cultures were placed in a growth chamber. Like these preliminary studies, immature embryo culture was used in the field experiment to test rapid generation skipping techniques with samples taken after induction hybridization.

For direct regeneration, MS immature embryos/seeds were transferred into MS medium. Then the explants were subcultured in Murashige and Skoog medium by planting in containers at 24 °C, 3000 lux, 60% humidity. For indirect regeneration, explants in CHU medium were kept for 1 week in the dark environment at 24 °C, 16/8 photoperiod conditions for callus formation. After the regeneration process, the somatic embryos (6 calli per/container) were subcultured in Murashige and Skoog medium by planting in containers at 24 °C, 3000 lux, 60% humidity. Colchicine (0.02%) was applied to ensure chromosome doubling in the calli formed by indirect regeneration method. Colchicine solution was prepared by dissolving in distilled water. This solution was then filtered through a 0.22 micrometer PTFE filter. After autoclaving of MS medium, colchicine was added to the tissue culture dishes in which the calli were to be placed.

**Determination of chromosome number by Feulgen method**

Structural and numerical analyses of chromosomes are important procedures and have been developed to make

chromosomes visible or to separate them with the help of special dyes and devices (Maluszynska, 2003). Chromosomes were analyzed using Feulgen stain to avoid any margin of error. In the study, samples that responded to direct regeneration were taken from the root meristems when the plants formed roots and Feulgen staining was applied. Digital images of the prepared slides were recorded by 100x magnification (Olympus, BX51TF, Japan), and chromosome counts were performed using ImageJ software (Rueden *et al*., 2021).

### Data analyses

This study was established with a completely randomized design with three replicates. In each replicate, 3-6 explants were subcultured depending on donor materials. The results obtained in the study were evaluated in the R package program (R Core Team, 2019). The variation of HIR values according to genotypes was presented in tables and graphs. The numerical values related to the results obtained in tissue culture studies were subjected to one-way analysis of variance and the means were compared using the Least Significance Difference test (LSD 5%).

## RESULTS AND DISCUSSION

### Comparison of haploid induction rates of donor materials

A total of 14577 seeds were evaluated and 662 of them were identified as haploid. Two of the donor materials (DON2, DON10) did not develop colouration after induction hybridization therefore haploid discrimination was not performed in their donors. For the remaining eight donor materials, HIR (haploid induction rate) values were determined by comparing the putative haploid seeds with the total number of seeds. As shown in Figure 2, HIR values for these materials ranged from 1.29 % to 7.12% (Table 1). However, it is noteworthy that significant differences were observed in the level of anthocyanin pigmentation used for haploid selection in donor materials. Among the inducer lines used in the study, CIM2-GTAIL-P2 line was found to have the highest potential for haploid seed production. The average HIR varied between 1.90% and 5.48%. Eder and Chalyk (2002) used MHI and M741H as inducer lines and found HIR values between 2.7 % and 8%. Our results agree with these findings.

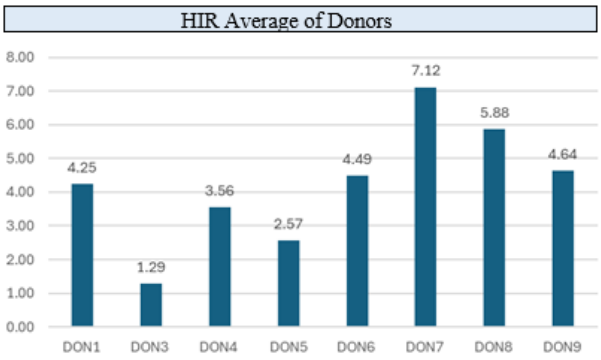


Figure 2. Variation of HIR values according to donor lines.

HIR values obtained from crosses with ADA1 inducer line were between 1.29% and 7.56 % and haploid seeds were obtained from eight donors crossed with this inducer line. Induction crosses made with CIM2GTAIL-P2 inducer line also showed HIR values between 1.96% and 8.54%. This inducer line allowed haploid seed formation in the crosses made with six donor populations. In the crosses made with Stock-6 inducer line, HIR values varied between 1.45% and 3.46% and haploid seeds were obtained from only three donors (Figure 3).

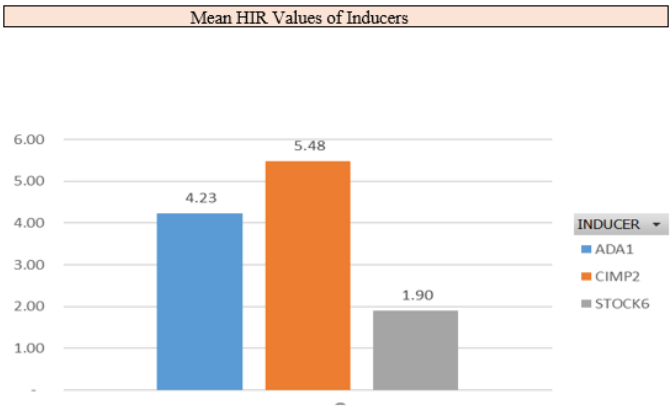


Figure 3. Variation of HIR values according to inducer lines.

In the previous studies in which HIR value was monitored, it was observed that there was a wide variation according to the donor material and inducer line used. In a study conducted under different climatic conditions, HIR varied between 2.17% and 5.33% (Hu, 2014). Cerit *et al*. (2016) reported a range of 1.28%-4.79%, Cengiz (2016) observed values between 17.17%-20.42%, Kahrıman *et al*. (2022b) reported a range of 9.2%-16.1%, Kahrıman *et al*. (2022c) recorded values between 6.08%-11.71%, and Taşkın (2023) found it to be 2.3%.

Table 1. HIR values obtained by induction hybridization in donor materials used in the study.

| Donors | ADA1 | CIM2GTAIL-P2 | STOCK-6 |
|---|---|---|---|
| DON1 | 4.35 | 5.49 | 2.90 |
| DON3 | 1.29 | - | - |
| DON4 | 2.84 | 4.57 | 3.26 |
| DON5 | 3.18 | 1.96 | - |
| DON6 | 4.49 | - | - |
| DON7 | 7.56 | 6.67 | - |
| DON8 | 6.10 | 5.65 | - |
| DON9 | 3.93 | 8.54 | 1.45 |
| Average | 4.22 | 5.48 | 1.90 |

In previous studies, HIR values ranging from 1.28 to 20.42% were reported. In our study, it can be said that there was wide variation in haploid seed formation for inducer lines according to donors. The main reason for this variation can be attributed to the genetic characteristics of donor materials and differences in their grain structure.

**Tissue culture studies**

**Direct regeneration**

Firstly, immature embryos were extracted from the seeds. Also, in preliminary studies, seeds were directly cultured on MS medium. The embryos were carefully transferred to the prepared MS medium without causing any damage. Prior to sowing, the sterile cabinet's UV light and ventilation were activated according to the general procedure, and the forceps and scalpel were sterilized using an autoclave. Then, the immature embryos were sown on MS medium. Direct seed or immature embryo explants provide plant formation in a short time (Figure 4). After 3 weeks of culture in light/dark photoperiod, plantlets were observed.

Barnabás *et al*. (1991) obtained successful results from direct regeneration method using immature embryo culture. In this study, positive results were obtained from direct regeneration method. Feulgen staining results of samples taken from the root tips of plants obtained by direct regeneration are shown in Figure 4. Cultured haploid specimens (no root and shoot colouration) were confirmed to be true haploids. In the study of Wan *et al.* (1989), in order to determine the ploidy level, tissues taken from the root tips and stained with aceto-carmine were examined under a microscope. In the current work the samples were examined under a microscope for chromosome counting and separation success was revealed (Figure 5). The Feulgen staining method used in our study showed that the ploidy levels of haploid materials can be verified. The results of the chromosome staining process confirmed the accuracy of haploid seed selection.

Figure 4. Plantlets obtained from direct regeneration method using seed and immature embryos.

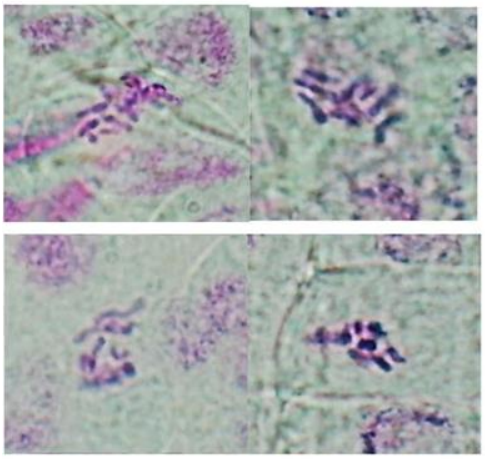

Figure 5. Chromosomes visualized in tissues taken from the root tips of haploid samples.

## Indirect regeneration

In indirect regeneration, it was aimed to ensure callus formation before immature embryos. Callus formation was observed after three weeks in this medium. However, only 4 out of 8 donors used in the study showed successful growth. Explants from other donors were excluded from tissue culture studies due to problems such as contamination and callus formation.

The four donors showed positive response to indirect regeneration. Callus formation was obtained from all these donor materials. The embryos were then separated in the callus mass and at this stage the embryo germinated. After three weeks of culture, callus germinated somatic embryos and visible shoots and leaves were observed. A plantlet originating from a somatic embryo growing on the callus was formed. The calli were subcultured in MS medium containing 0.02% colchicine. During subculture, 6 calli were subcultured. And 3-6 calluses were formed according to donor materials. Callus production rates ranged from 50% to %100. The number of somatic embryos formed on these calli varied between 1.0 to 2.3 and the frequency of embryogenic callus production was found to be between 25% and 58.3% (Table 2, Figure 6). The calli containing the formed somatic embryos were transferred to standard MS medium within 7 days and plant formation was observed.

Table 2. Number of callus and somatic embryos obtained by indirect regeneration method in the study.

| Donor | Callus Number and Ratio | Somatic Embryo Number and Ratio |
|---|---|---|
| DON3 | 5 b (83.3%) | 2.3 (58.3%) |
| DON4 | 3 d (50%) | 1.3 (50.3%) |
| DON6 | 6 a (100%) | 2.0 (50%) |
| DON7 | 4 c (66.7%) | 1.0 (25%) |

In this step, colchicine was applied to the callus obtained from immature embryo culture for chromosome doubling. It is thought that colchicine application to embryogenic tissue *in vitro* is more effective to achieve chromosome doubling (Barnabas *et al*., 1999). Furini and Jewel (1995) used five selfed maize and *Tripsacum dactyloieds* hybrids. In addition, the frequency of embryogenic callus production was found between 14.3% and 66.6%. The highest percentage of chromosome doubling was observed in calli treated with colchicine (Petersen *et al*., 2002). Therefore, 0.02% colchicine solution was used for chromosome doubling in our study. All these procedures were carried out under laboratory conditions in a laminar flow hood protected against any kind of contamination. It was possible to continue the line development process by placing the plants obtained from indirect regeneration under controlled conditions after acclimatization.

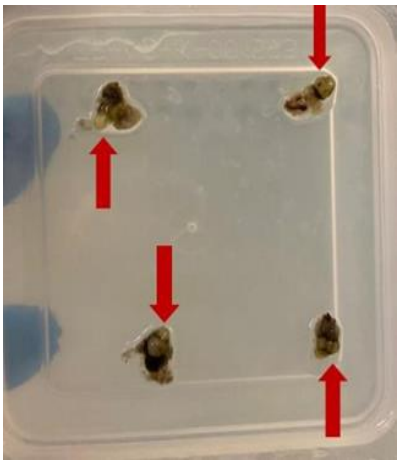

Figure 6. Development of somatic embryos in explants treated with colchicine

## CONCLUSION

The results of the study were analyzed under two main topics: haploid seed formation and integration of *in vitro* method *in vivo* double haploid technique.

HIR values determined for haploids in donor materials varied between 1.29 % and 7.12%. No haploid seed formation was observed in two donors crossed with inducer lines. The highest HIR value was obtained in the CIM2-GTAIL-P2 line. The results revealed that haploid seed formation varied depending on genotypic effects.

Immature embryo culture is widely used in cereals such as wheat and offers advantages in maize breeding. In this study, the integration of immature embryo culture with direct regeneration and callus culture methods was investigated. While 4 out of 8 donor materials responded positively in the first approach, 4 materials formed callus in the second method. Plant formation was observed after chromosome doubling.

While it takes 12-19 months to obtain seeds in classical *in vivo* methods, this period can be reduced to 8-9 months with integration of immature embryo culture to classical *in vivo* maternal haploid technique. This method saves 3-4 months of time. Moreover, it is possible to obtain more dihaploid clones with immature embryo culture. However, indirect regeneration method failed in some donor materials. The importance of colouration in early seed separation was also important for success in the accurate discrimination of haploids.

## ACKNOWLEDGEMENT

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