The Effect of Different 2, 4-D Doses on Callus Induction and Chromosomal Structure in Maize (Zea Mays L.)

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Abstract— *This research in which the matured embryos of 10 registered common corn varieties grown in Turkey (Pioneer 31N27, Pioneer 31P41, Pioneer 3223, Pioneer 34N24, ADA 8924, DKC 6022, BC 666, TECTOR, ADA 523 and HELEN) were used as plant materials was carried out in Biotechnology Laboratory, Department of Field Crops, Faculty of Agriculture, Ankara University, Turkey. In the stage of in vitro of this research which was arranged in vitro and in vivo media, the effect on callus induction and plant regeneration in 6 different varieties of 2,4-D doses (0, 2, 4, 6, 8 and 10 mg/l) through callus culture was investigated. In the stage of in vivo, samples from the root tips composed of the callus of all maize varieties were taken, and the changes observed in the chromosomal numbers in maize lines were evaluated. As a result of the microscopic analyses of the root tips obtained through the application of 2,4-D auxin in different corn varieties in different doses, changes were observed in the chromosomal numbers of Pioneer 31N27 (2n=18), Pioneer 3223 (2n=19) and Pioneer 34N24 (2n=19) maize varieties. Mitotic anomaly observed through the application of 2,4-D was established as aneuploidy (the change of the chromosomal number in genome) and it was found to form as the decrease of the chromosomal number (hipoploidy). According to the data found, the dose of 2,4-D to be used in the transgenic maize producing studies to be utilized in Turkey was determined to be 2 mg/l providing the highest callus formation ratio but causing no chromosomal deviations.*

Keywords— Zea mays, Corn, Maize, Callus Induction, Chromosomal Number.

I. INTRODUCTION

Maize (*Zea mays* L.) (2n=2x=20), which is grown widely in the world, industrialized and because it has rich nutrients, is very valuable and is a field plant which has usage varieties for both human and animal nutrition. Maize is the grain which has the highest yield in all cool and warm climate grain in the world and which uses the solar energy best.

Maize is used both in human nutrition directly and in the industry of starch, glucose, oil and dry food as raw material and it is a very renewable energy source. Most of the maize product in the world is used as animal food [1].

As well as clasical studies of breeding nowadays, significant developments were established also in related studies with genetically modified maize obtained through various methods such as particle bombardment [2], [3], [4] and *Agrobacterium* infection [5], [6].

The first molecular-marker maize map was published in 1986 [7]. It contains 116 loci and in this mapping, random genomic clones like cDNA and probes were used. Genetic linkage maps of maize consist of thousands of Classical Mutation Loci, Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeat (SSR), Quantitative Trait Loci (QTL) and Expressed Sequence Tags (EST) markers [8], [9], [10], [11].

Plant biotechnology studies, which started in the 1990s in Turkey focused on tissue culture at the beginning and began to entegrate with molecular biology and genetical technics in the 2000s. Most of the researchers related with plant biotechnology are on tissue culture. The plant regeneration from tissue culture of mazie was first reported in 1975 [12].

The auxin, a group of natural growth regulators, support rooting, cell development and callus induction. The widely used auxin group hormone to support embryogenesis is a pestiside, 2,4-Dichlorophenoxy-acetic acid (2,4-D). 2,4-D chemical is frequently preffered to start callus induction. However, a chemical agent 2,4-D has a powerful immunotoxic effect. 2,4-D increases the risk of autoimmune reaction and causes an increase in the alergenic reaction. In addition to above-mentioned effects, 2,4-D is known to have teratogenic effects.

The 2,4-D auxin is catalysed with 2,4-D/2-Oxoglutarate dioxygenase and turns into 2,4-Diclorophenol (DCP). DCP (a chemical agent) in human and animals skin inflammation, liver damage and it causes mortality in high doses.

The main aim of the study is the determination of 2,4-D doses which may be used to produce transgenic maize in Turkey, which provide the highest callus induction ratio and which don't form chromosomal deviations. Therefore, different 2,4-D doses were applied to different origion maize varieties commonly planted in Turkey and the most effective but chromosomally the most harmless callus induction protocol.

II. MATERIALS AND METHODS

2.1. Plant Materials

This research in which the matured embryos of 10 registered common corn varieties grown in Turkey (Pioneer 31N27, Pioneer 31P41, Pioneer 3223, Pioneer 34N24, ADA 8924, DKC 6022, BC 666, TECTOR, ADA 523 and HELEN) were used as plant materials was carried out in Biotechnology Laboratory, Department of Field Crops, Faculty of Agriculture, Ankara University, Turkey.

2.2. Sterilization of Equipments

All the studies for in vitro culture were carried out inside a laminar air flow cabinet in aseptic conditions by using sterilized equipments, plant materials, glass materials and chemicals. The hood surface was wiped clean with paper towel soaked in ethanol and sterilized by germicidal ultraviolet light for at least 10 minutes prior to usage. The instruments and glasswares were sterilized in autoclave at 121°C with 15 psi for 30 minutes and then dried in the oven.

2.3. Surface Sterilization of Explants

The surface sterilization of mature seeds was performed in fully sterilized glasswares inside the sterilized laminar air flow cabinet. All seeds were surface sterilized with %70 ethanol for 5 min. in magnetic shaker and the seeds were washed thoroughly 3 times with sterile distelled water. Each shake was completed in 30 sec. during rinsing. Then, the seeds were shaken in magnetic shaker for 35 min. in 5% (v/v) commercial sodium hypocloride on which it includes a few drops of Tween 20. the seeds were washed thoroughly 7 times with sterile distelled water. The seeds put in the sterile distelled water were swolled in water bath (35°C) for 2 days.

2.4. Preparation of Culture Media

Petri dishes contain MS [13], saccarose, NaCl, agar ve 2,4-D for callus induction and petri dishes contains MS, saccarose, NaCl, agar for callus development and regeneration.

2.5. Callus Induction and Development

The embryos which induced callus in the sterilized laminar air flow cabinet were removed from the endosperm with the surgical blade and they were put in the solid MS medium containing 2,4-D doses in different concentrations of 0, 2, 4, 6, 8 ve 10 mg/l. The culture media (pH 5.8) was autoclaved at 121°C for 20 min. (1.2 psi). The endosperm hoollowed and streched-petri dishes were reweighed and the difference between them was the "callus weight (g)". The numbers of the embryos inducing callus were established and it was used in the determination "callus induction (%) value".

The cultures including calli were incubated in the dark at 26°C for 21 days for callus induction. The study was built on random sample of parcels in 3 repeats in the petri dishes containing 10 embryos for each genotype (Fig.1).

2.6. Cytological Studies

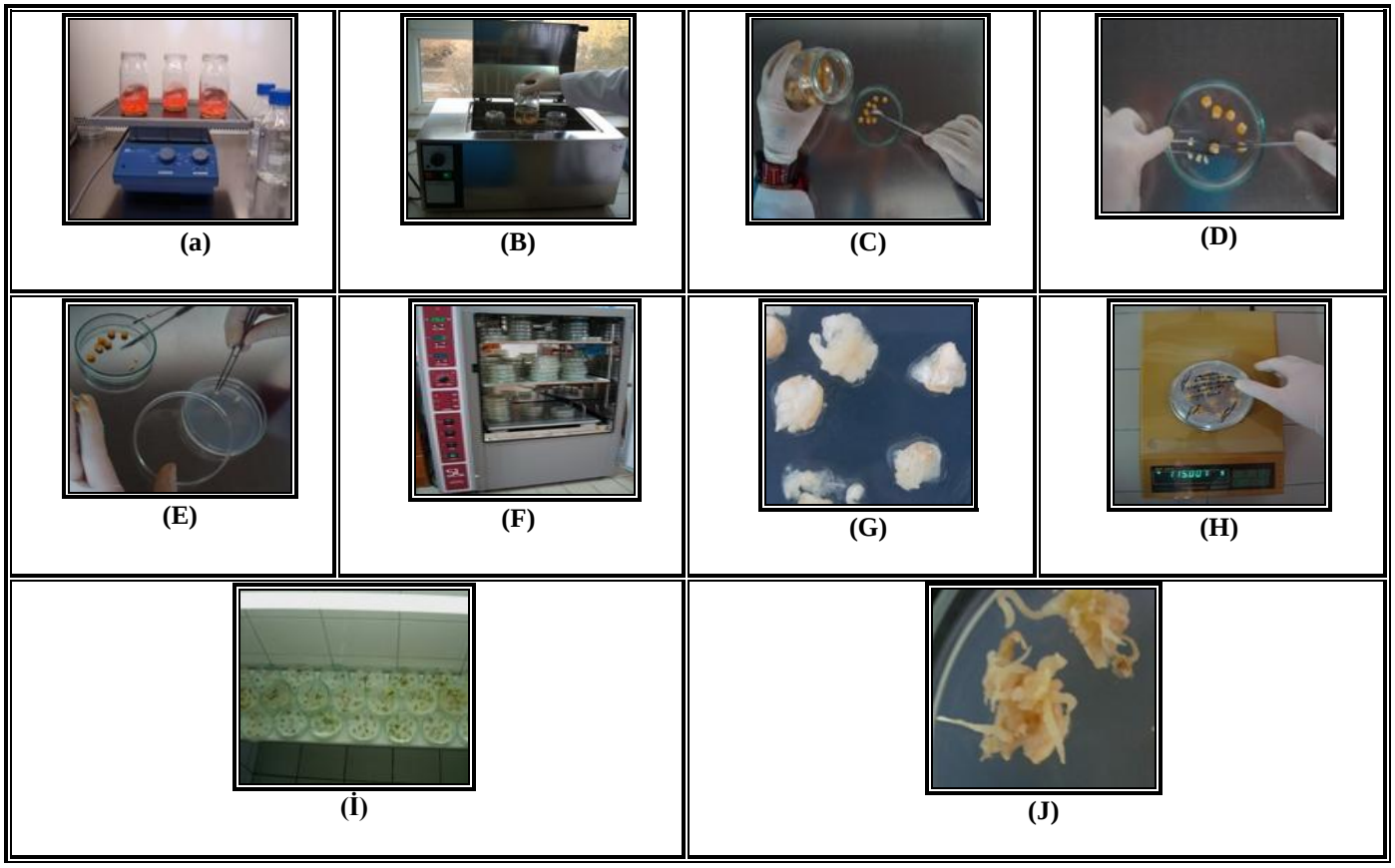
Root tips samples composed of from the calli of all maize varieties were taken and the changes forming in the chromosomal numbers of maize were investigated.

2.7. Prefixation

The α-monobromonaphtaline application to the root tips cut with the surgical blade is the first procedure applied to the material. Since α-monobromonaphtaline cannot dissolve in water, 1% α-monobromonaphtaline stock solution was prepared.

2.8. Fixation

The roots taken from the refrigerator were washed with the distelled water several times and the best result was obtained placing them in 99% acetic acid for 30-60 min.



(A) SURFACE STERILIZATION OF MAIZE.
(B) SOFTENING OF THE MAIZE SEEDS IN WATER BATH.
(C) TRANSFER OF MAIZE SEEDS TO THE PETRI DISHES INSIDE THE STERILIZED LAMINAR AIR FLOW CABINET.
(D) REMOVAL OF MAIZE EMBRYOS WITH THE SURGICAL BLADES INSIDE THE STERILIZED LAMINAR AIR FLOW CABINET.
(E) TRANSFER OF MAIZE EMBRYOS INTO MS MEDIUM.
(F) PLACING OF THE EMBRYO TRANSFERRED PETRI DISHES IN THE DARK INCUBATOR (3 WK).
(G) CALLUS INDUCTION.
(H) WEIGHING OF THE PETRI DISHES INCLUDING CALLUS.
(I) PLACING OF THE CALLI TRANSFERRED INTO HORMON-FREE PETRI DISHES IN THE CULTURE ROOM (~4 WK).
(J) ROOT FORMATION FROM THE CALLI OBTAINED FROM MATURE EMBRYOS.

FIG 1. ROOT FORMATION FROM MAIZE CALLUS.

2.9. Hydrolysis

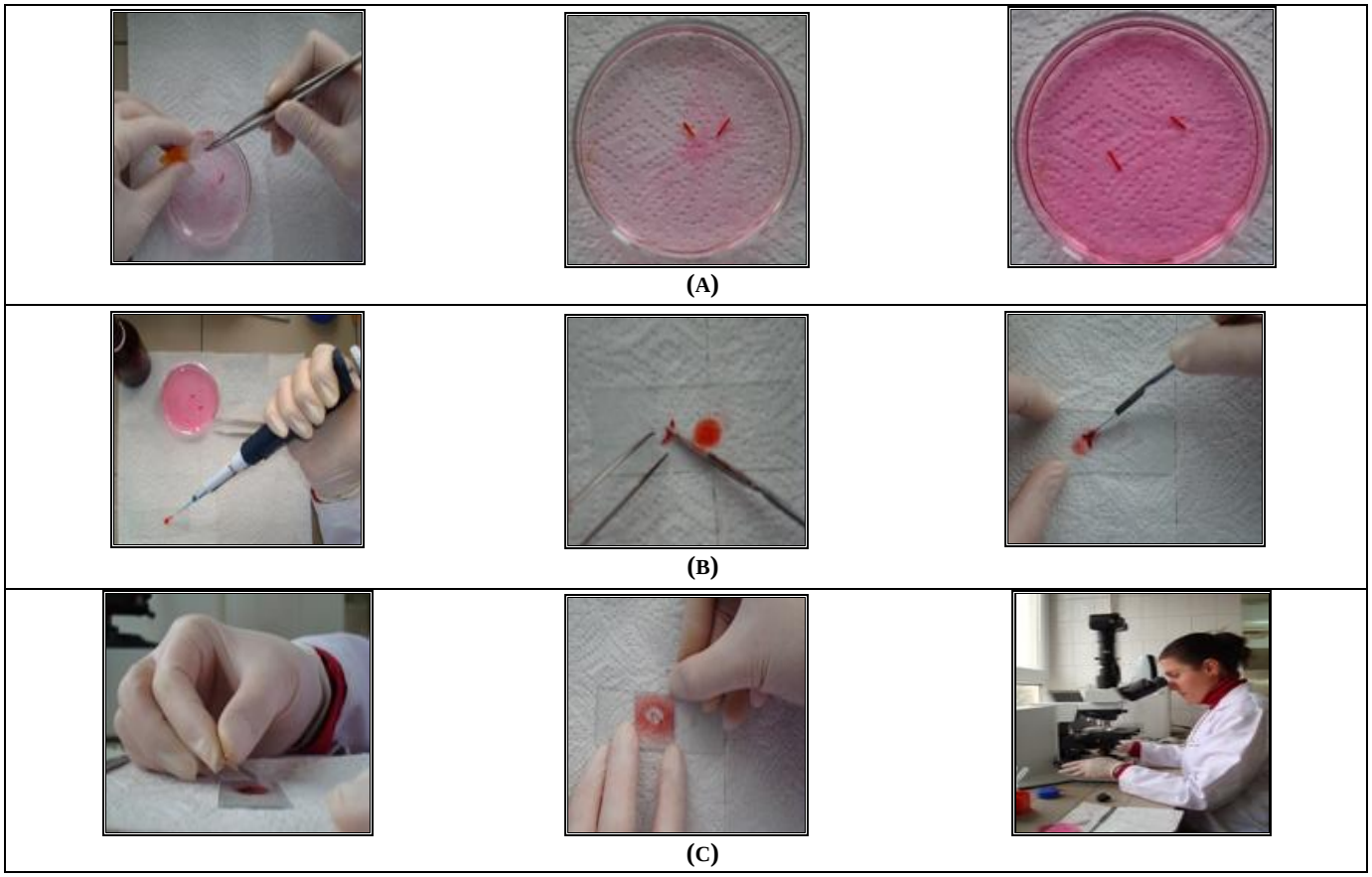
It is the phase in which the roots were placed in the water bath in 1 N HCl. The period of hydrolysis was established as 10 min., the period in which root tips were best stained.

2.10. Staining

Chromosomes were stained with feulgen and aseto-carmin stain [14].

2.11. Microscopical investigation of the root tips

The root tips in Feulgen stain, were placed in the room temperature and in the dark for 30 min. Then, they were transferred into the horizontal glassware together with the stain and the glassware was passed through alcohol flame a few times. After the staining procedure, all the roots were seen to have been stained red violette. The 1-2 mm length tips stained with dark purple of the root tips were cut from the light coloured parts. This 1-2 mm length tip in which meristem cells lie was the part used in the preparats prepared for the microscopic observation (Fig 2.).



(A) THE TRANSFER OF THE ROOT TIPS STAINED WITH FEULGEN STAIN INTO DESTILLED WATER.
(B) CUTTING THE 1-2 MM LENGTH TIPS STAINED WITH DARK PURPLE OF THE ROOT TIPS FROM THE LIGHT COLOURED PARTS. DIVIDING THE DARK PURPLE COLOURED PART INTO SMALL PARTS AND STAINING WITH THE ACETO-CARMINE STAIN.
(C) THE PREPARATION OF THE PREPARAT AND INVESTIGATION IN THE MICROSCOPE.

FIG.2. THE MICROSCOPIC INVESTIGATION OF ROOT TIPS.

2.12. Data Evaluation

The variance and correlation analysis of the data were evaluated with SPSS Statistical Package Programme, the most suitable package programme.

III. RESULTS AND DISCUSSION

According to the research findings, callus induction was observed in the mature maize embryos in MS media without 2,4-D. Therefore, also in another brand MS media callus induction in mature maize embryos without 2,4-D. At the end of the studies performed, callus induction in mature maize embryos was observed in the medium without 2,4-D prepared with the second brand MS.

At the end of the studies of maize tissue culture, the IAA ratio in the structure of maize was determined to be high [15], [16], [17], [18]. For this reason, eventhough MS media doesn't contain auxin, in high nutrient medium callus induction in culture conditions in the maize can be observed. The results obtained in our study are similar to the above-mentioned studies.

It was found that the effect of the amount of 2,4-D was very significant in callus induction and callus weight ($p<0,01$). Reviewing the Duncan Grouping Data obtained, it was determined that the ratio of callus weight and callus induction changed depending on the amount of 2,4-D.

In our study, it was determined that the effect of genotypes in callus induction was significant ($p<0,01$). Similar findings were obtained in a lot of studies performed in this subject [19], [20], [21], [22]. Moreover, the differences among the genotypes because of the callus induction were reported to occur due to the differences in plant hormon level [23], [24].

At the end of the correlation analysis performed among the characters in mature maize embryo culture, a medium level, negative and significant relation was seen to exist between the amount of 2,4-D and the callus induction ratio (r= -0,247, p≤0,05). Accordingly, as the amount of 2,4-D increased, the callus induction decreased. A medium level, negative and significant relation was seen between the amount of 2,4-D and shoot development ratio (r= -0,441, p≤0,05). Therefore, as the amount of 2,4-D increased, the development of the shoot decreased.

A medium level, negative and significant relation was seen between callus weight and shoot development ratio in somatic embryos (r= -0,338, p≤0,05). Accordingly, as the development of the shoot increased, callus weigth decreased.

Similarly, a medium level, negative and significant relation was seen to exist between the amount of 2,4-D and the ratio of callus weight (r= -0,457, p≤0,05). Therefore, as the amount of 2,4-D increased, callus weigth decreased. Howeverei there was no relation between the ratio of callus induction and callus weight.

Also, at the end of the mature and immature embryo culture of the wheat study, the relation between the ratio of callus induction and callus weight was not significant [20].

In this study, at the end of the microscopic analysis of the root tips obtained with the application of the 2,4-D auxin to different maize varieties in different doses, a deviation (aneuplody and hipoploidy) was observed in the chromosomal numbers of Pioneer 31N27 (2n=18) (Fig.3), Pioneer 3223 (2n=19) and Pioneer 34N24 (2n=19) maize varieties threated with 10 mg 2,4-D; however, a deviation was not observed in the chromosomal numbers of the maize varieties threated with the other different doses of 2,4-D.

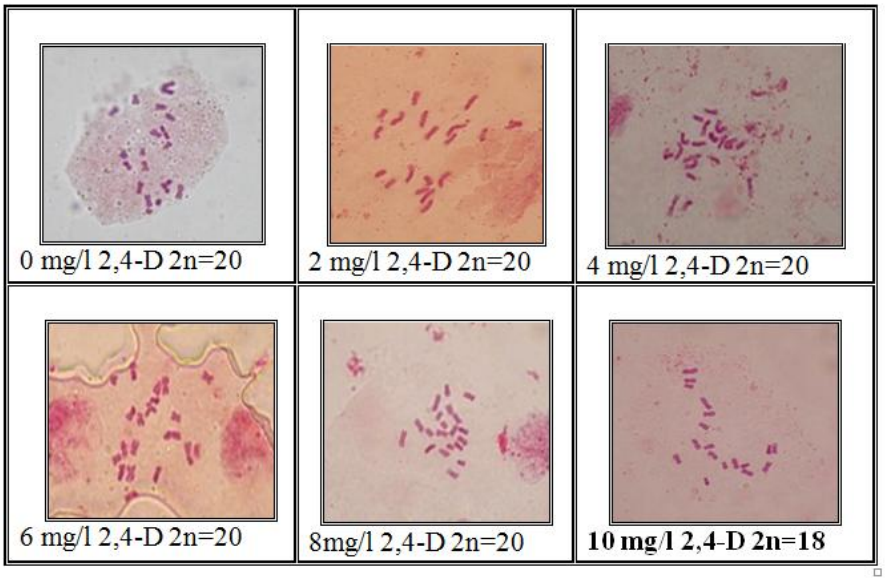


FIG 3. SOMATIC CHROMOSOMES OF PIONEER 31N27 MAIZE VARIETY THREATED WITH DIFFERENT 2,4-D DOSES.

Just like in our study, 2,4-D auxin was the reason of genetical abnormalities [25], [26] like aneuploidy. Such abnormalities were reported to have been caused from the effect of the pesticide on DNA [27].

The variation of chromosomal number observed in this study is considered to occur because of the changes in DNA methylation. As, DNA has got a high methylation, gene activity is suppressed. For example, it was reported that 2,4-D caused DNA methylation in carret cell culture and therefore carrot culture didn't develop [28].

To sum up, at the end of the application of 2 mg/l 2,4-D in maize, the values of the highest callus induction and callus weight were found and also, in the chromosomal numbers of the maize threated with 2mg/l 2,4-D no abnormalities were observed.

IV. CONCLUSION

In our research, data related with callus, the ratio of shoot induction and callus weigth were obtained. In the 10 maize varities studied the highest callus induction was observed in HELEN (100%) maize variety threated with 2 mg/l 2,4-D and the variety of the maize in which callus weight was the most was established to be DKC 6022 (2.85 g) threated with 2 mg/l 2,4-D.

In this research, at the end of the high dose application of 2,4-D, the variation of chromosomal number in *Zea mays*, that is, chromosomal abberrations occurred. Such mitotic abnormalities were reported to have been caused from the effect of the pesticide on DNA. The toxic effects of the pesticides on different living systems were reported with a lot of studies.

For this reason, this commonly-used 2,4-D chemical must be used carefully in high doses because it may be fatal in several organisms including human.

In conclusion, different 2,4-D doses were applied to different origion maize varieties commonly planted in Turkey and the most effective but chromosomally the most harmless callus induction protocol was determined. This protocol can also be used in the studies to produce transgenic maize in Turkey in the future.

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