





Article

Impact of Ionic Liquids on Induction of Wheat Microspore Embryogenesis and Plant Regeneration

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Abstract: Ionic liquids are novel compounds with unique chemical and physical properties. They can be received based on synthetic auxins like 2,4-dichlorophenoxyacetic acid or dicamba, which are commonly used hormones in microspore embryogenesis. Nevertheless, ionic liquids have not been adapted in plant in vitro culture thus far. Therefore, we studied the impact of ionic liquids on the ability to undergo microspore embryogenesis in anther cultures of wheat. Two embryogenic and two recalcitrant genotypes were used for this study. Ten combinations of ionic liquids and 2,4-dichlorophenoxyacetic acid were added to the induction medium. In most cases, they stimulated induction of microspore embryogenesis and green plant regeneration more than a control medium supplemented with only 2,4-dichlorophenoxyacetic acid. Two treatments were the most favorable, resulting in over two times greater efficiency of microspore embryogenesis induction in comparison to the control. The effect of breaking down the genotype recalcitrance (manifested by green plant formation) was observed under the influence of 5 ionic liquids treatments. Summing up, ionic liquids had a positive impact on microspore embryogenesis induction and green plant regeneration, increasing the efficiency of these phenomena in both embryogenic and recalcitrant genotypes. Herbicidal ionic liquids can be successfully used in in vitro cultures.

Keywords: ionic liquids; auxin-like treatment; induction of embryogenesis; microspore embryogenesis; haploid; wheat

1. Introduction

Microspore embryogenesis (ME) is a very attractive, quick and efficient method for obtaining fully homozygous plants. Haploids or doubled haploids are regenerated from immature anthers or isolated microspores. This process is called androgenesis or pollen embryogenesis. Haploids have just one set of genes, which makes them the perfect target for genetic improvement via transformation [1] or genetic engineering [2]. Moreover, they can easily be treated with chemical compounds leading to non-disjunction of chromosomes during mitosis, for example, with colchicine, thereafter giving rise to homozygous plants—called doubled haploids—in a single generation. Therefore, it visibly shortens the time needed to obtain homozygosity as compared to conventional methods [3]. Doubled haploid lines are valuable plant material for genetic studies and plant breeding [4].

The application of ME in breeding programs has great agronomic importance, not only because it results in fast achievement of genetic homozygosity but also because it maintains useful recessive genes, which are of primary importance in the production of new breeding lines and varieties [5].

This technique makes it possible to create new gene combinations resulting in high yielding varieties, resistance to diseases and pests and higher tolerance to external factors [6]. For the successful application of ME, a large number of homozygous diploid plants are needed [7]. Unfortunately, the potential of ME is not yet fully utilized in wheat due to the low efficiency of microspore induction to embryogenic structure formation and plant regeneration. In addition, many wheat genotypes are characterized by genotypic recalcitrance, which significantly limits the effectiveness of this method in the production of new varieties. For this reason, it is important to find effective inducers which may break this genotypic recalcitrance, increase embryogenicity and doubled haploid yield.

The induction of ME is a complex epigenetic process. External factors like stress and/or hormonal signals may release epigenetically silenced genes [8]. The induction of ME initiates a cascade of events in which the generative nature of microspores becomes sporophytic. The use of proper stress factors makes microspores undergo symmetrical divisions characteristic of somatic cells. The application of exogenous auxins to the induction medium is known to be a stimulator of that process. Their effect is similar to that of indolyl-3-acetic acid (IAA), which is a natural hormone regulating plant growth and development [9]. Unfortunately, recalcitrant wheat genotypes do not undergo induction under the influence of common growth regulators. The lack of universal and effective ME inducers was the motivation for the search of new compounds that could potentially increase the number of embryogenic structures in androgenesis.

Ionic liquids (ILs) are novel chemical compounds with special properties, defined as ionic-chemical compounds with a melting point below 100 °C. They are safe solvents, allow for efficient use of energy and are used in catalytic reactions. This property most often comes with a significant difference in size between a large organic cation with an asymmetrical structure and a small organic or inorganic anion [10]. ILs are beneficial in for example, chemical synthesis, catalysis, extraction, biomass conversion, fuel production and processing, liquid crystal development, biotransformation, biotechnology and electrochemistry [11–13].

Third-generation ILs have targeted biological, physical and chemical properties and are used as pharmaceutical ingredients or in agriculture [14,15]. In terms of agricultural use, ILs have been documented as inducers of systemic acquired resistance, which was tested on model plants *Nicotiana tabacum* var. *Xanthi* infected with Tobacco mosaic virus (TMV) [16–18]. Other tests also showed the potential usage of ILs as locally acting antibacterial agents, stimulating natural defense systems in plants [14]. Third-generation ILs have documented herbicidal properties, for example, against gallant soldier (*Galinsoga parviflora*), white goosefoot (*Chenopodium album*), common sorrel (*Rumex acetosa*), cornflower (*Centaurea cyanus*) and white mustard (*Sinapis alba*) [19,20]. Some studies indicate the phytotoxicity of ILs depends on the plant species [11,12]. As reported, using the lowest concentration of ILs causes only minor changes in plant phenotype and may stimulate development and growth instead [20]. In wheat, ILs have been considered as solvents for biocatalysis (Zhao, 2010) and are used for wheat straw pre-treatment having the potential to produce bioethanol (Li et al., 2009). Herbicidal ILs are laboratory-obtained derivatives of commonly used synthetic plant hormones, for example, dicamba or 2,4-dichlorophenoxyacetic acid (2,4-D), also used as herbicides when dosed in high concentrations [15,16]. This leads to the conclusion that it is worth testing ILs synthetic hormones in in vitro plant culture.

Therefore, in the present study we examined the impact of ILs on the induction of microspore embryogenesis and plant regeneration in anther culture of spring wheat.

2. Results

In total 24,000 anthers were analyzed in the experiment-300 anthers/genotype/ILs treatment/year. IL addition to the induction medium and the genotype of donor plants had a statistically significant effect on the number of embryogenic structures (ES), green plants (GP) and albino plants (AP). However, there was no statistically significant effect of the year on the above parameters. The data analysis is presented in Table 1.

Table 1. The effect of year, treatment and genotype on the number of: embryogenic structures (ES), green plant (GP) and albino plant (AP) regeneration.

Source	Df	No. of ES		No. of GP		No. of AP	
		MSq	p-Value	MSq	p-Value	MSq	p-Value
Year	1	0.3	0.921	3.20	0.43	0.622	0.432
Treatment	9	156.7	<0.001 ***	25.99	<0.001 ***	15.3	<0.001 ***
Genotype	3	2169.2	<0.001 ***	187.78	<0.001 ***	121.42	<0.001 ***
Residuals	66	31.8		5.08		3.94	

Signs: Source-source of variation, Df-degrees of freedom, Msq-mean square. Signif. codes: '***' < 0.001.

2.1. Influence of ILs and Genotypes on Microspore Embryogenesis Induction

Embryogenesis induction of uninucleate microspores (Figure 1A) was observed in all analyzed experimental treatments, which resulted in 873 ES in total (Figure 1B). PCA analysis illustrated the capability of selected cultivars to undergo embryogenic induction under the effects of ILs (Figure 1C). The sum of Dim1 and Dim2 indicated that the percentage of information from the original data contained on the biplot was 84.8%. Such analysis lead to the formation of two distinct genotype groups which responded differently to the applied regulators.

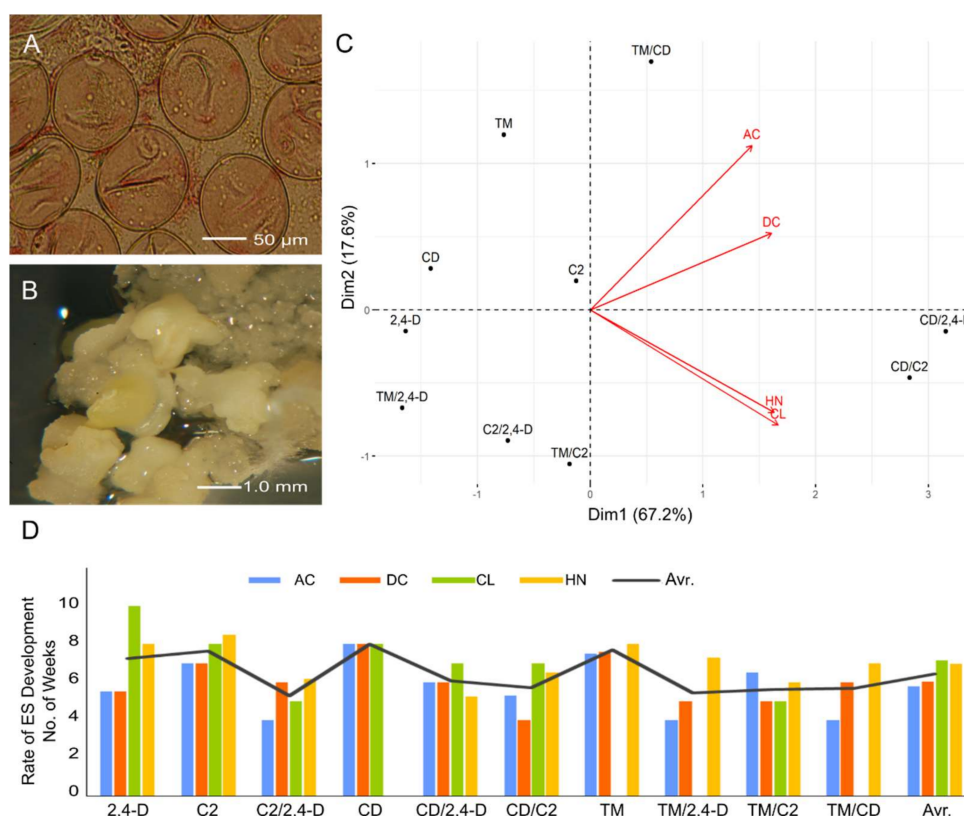


Figure 1. Influence of ionic liquids and genotypes on microspore embryogenesis induction. (A) Microspores in uninucleate stage required for anther culture. Bars = 50 µm. (B) Embryogenic structures (ES) obtained on induction media. Bars = 1.0 mm. (C) Principal component analysis (PCA) biplot analysis showing the interaction of genotypes (AC, DC, CL, HN) and treatment of ILs and/or 2,4-D in induction media (2,4-D; C2; CD/2,4-D; CD; CD/C2; C2/2,4-D; TM; TM/2,4-D; TM/C2; TM/CD). (D) Rate of embryogenic structures (ES) development of analyzed genotypes under treatment of ILs and/or 2,4-D. 2,4-D–(2,4-dichlorophenoxyacetic acid). C2–(2-chloroethyl ammonium 2,4-dichlorophenoxy acetate). CD–(2-Chloroethyl trimethylammonium 2,6-dichloro-2-methoxybenzoate). TM–(Trimethylvinylammonium (2,4-dichlorophenoxy) acetate).

AC and DC varieties, characterized by 7-day quicker ES regeneration on average, in comparison to CL and HN cultivars (Figure 1D). The first ES were observed 4 weeks after the anther culture was established. ES growth rate depended on the donor plant's genotype. The second crucial factor was treatment. The C2/2,4-D medium stimulated ES the quickest—it took only 5 weeks on average for the structures to achieve a diameter of 0.5 cm.

AC and DC varieties were also more effective in embryogenic structure formation (ESF), compared to CL and HN cultivars (Table 2). ILs' effect on the microspores of specific genotypes was individual (Figure 2A–D). CD/2,4-D and CD/C2 treatments stimulated the induction of microspore embryogenesis the most effectively among all of the analyzed cultivars. The average ESF on those mediums was 6.0 ES/100 plated anthers (Table 2). The highest efficiency of microspore embryogenesis induction (14.17 ES/100 placed anthers) was observed on the CD/C2 medium in anthers of DC variety. The TM/CD treatment had the highest induction potential of ME mainly on the embryogenic cultivars. This effect was not observed among the cultures of recalcitrant CL variety (Figure 2C). Low ESF levels in both recalcitrant genotypes must be noted on the control medium, as the ESF level did not exceed 0.2 ES/100placed anthers. This means that treatment with most ILs stimulated microspores to produce ES better than only 2,4-D (Figure 2C,D). This relationship could also be observed in the response of embryogenic genotypes (Figure 2A,B).

Table 2. Impact of ionic liquids and genotypes on efficiency of embryo structures formation (ESF).

	Treatment									
	2,4-D	C2	C2/2,4-D	CD	CD/2,4-D	CD/C2	TM	TM/2,4-D	TM/C2	TM/CD
Genotype	ESF									
AC ^b	3.8	5.5	4.5	4.5	9.2	6.3	6.8	1.7	4.3	8.5
DC ^a	4.7	8.0	3.3	5.8	10.7	14.2	7.0	6.8	5.2	10.2
CL ^c	0.2	0.5	0.2	0.2	1.5	1.7	0.0	0.0	1.0	0.0
HN ^c	0.2	0.7	1.7	0	2.5	1.8	0.2	0.5	1.0	0.8
Avr.	2.2	3.7	2.4	2.6	6.0	6.0	3.5	2.3	2.9	4.9

ESF—no. of embryogenic structures/100 placed anthers. Groups followed by the same letter are not statistically significant by Tukey test ($p < 0.05$). 2,4-D—(2,4-dichlorophenoxyacetic acid). C2—(2-chloroethyl ammonium 2,4-dichlorophenoxy acetate). CD—(2-Chloroethyl trimethylammonium 2,6-dichloro-2-methoxybenzoate). TM—(Trimethylvinylammonium (2,4-dichlorophenoxy) acetate).

Applying two different growth regulators to the medium has a beneficial effect on ESF in most treatments (Figure 2). In case of the recalcitrant genotypes, the simultaneous action of two regulators was crucial to effective microspore induction embryogenesis. Moreover such treatment of anthers resulted in quicker ES growth (Figure 1D).

2.2. Influence of ILs and Genotypes on Plant Regeneration

Depending on the applied IL treatment, varying efficiencies of green plant regeneration (GPR) and albino plant regeneration (APR) were observed (Figure 3). A large variation of the regeneration level was observed in specific experimental combinations. Moreover a high effectiveness of regeneration was not uncommon in single trials as shown by the outliers (Figures 3 and 4). Most IL treatments increased the GPR of embryogenic genotypes in comparison to regeneration on control medium. Moreover 5 treatments of ILs caused regeneration in plants of the recalcitrant cultivars, which on the contrary was not observed in the medium containing only 2,4-D (Figure 4; Table 3).

The medium with addition of CD/2,4-D was the best in stimulating the development of green plants. The value of GPR of both of the embryonic genotypes on that medium was over twice as high in comparison to the control. Moreover the highest yield of GP from anthers of recalcitrant variety was collected (Table 3). The average GPR for all the genotypes altogether on CD/2,4-D medium was equal to 2.5 GP/100 placed anthers. For comparison, the AC value of this parameter reached 6.0 GP/100 placed anthers. In addition two other experimental treatments-CD/C2 and TM—were favorable for

embryogenic genotypes. From anthers of AC and DC cultivars on those media, over two times as many green plants were obtained in comparison to the control.

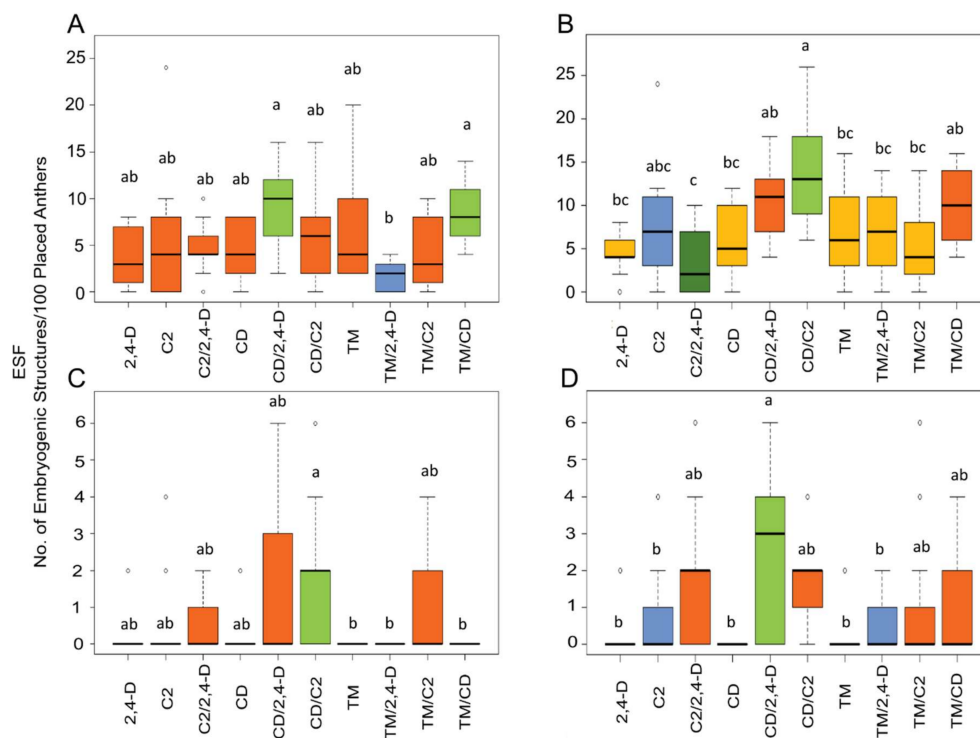


Figure 2. Boxplots of the effect of ionic liquids (ILs) on efficiency of embryogenic structures formation (ESF) in analyzed genotypes in comparison to control media containing 2,4-D: (A) embryogenic cultivar AC; (B) embryogenic cultivar DC; (C) recalcitrant cultivar CL; (D) recalcitrant cultivar HN. Groups followed by the same letter are not statistically significant by Tukey test ($p < 0.05$). Middle quartile means median.

The medium supplemented by CD/C2 efficiently stimulated microspores to undergo embryogenesis induction, however the obtained plants were mainly albino (Figure 5). Most of the albino plants from anthers with the genotype DC, CL, HN were collected from the medium supplemented with that combination of regulators. Anthers of DC genotype regenerated albino plants more often than other analyzed cultivars (Table 3). Although DC cultivars were characterized by the highest efficiency of embryogenesis induction, a significant part of the obtained ES were plants with chlorophyll defects, which further led to decrease of GPR.

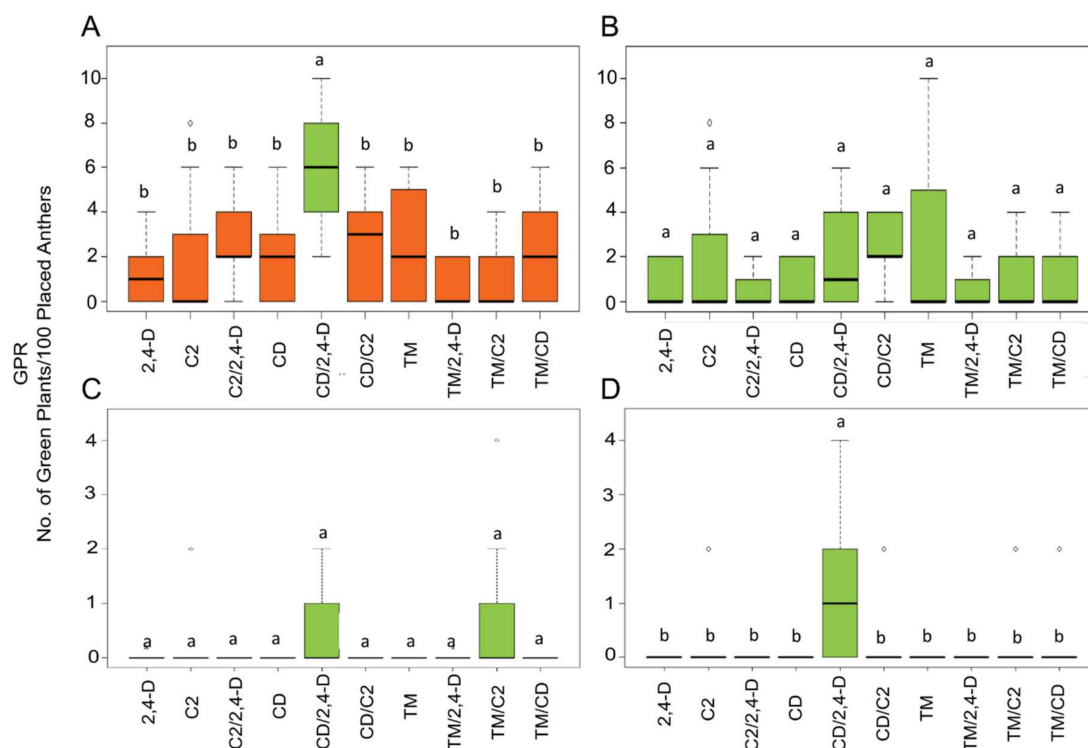


Figure 3. Plants regeneration in anther culture of wheat: (A) Green plant; (B) Albino plant. Bars = 2.0 mm.

Table 3. Impact of ionic liquids and genotypes on efficiency of green plant regeneration (GPR), albino plant regeneration (APR) and green plant frequency (GPF).

Genotype	Treatment									
	2,4-D	C2	C2/2,4-D	CD	CD/2,4-D	CD/C2	TM	TM/2,4-D	TM/C2	TM/CD
GPR										
AC ^a	1.2	1.7	3.0	1.8	6.0	2.7	2.5	0.8	0.8	2.2
DC ^b	0.8	1.8	0.5	0.8	2.2	2.5	2.5	0.5	1.0	1.0
CL ^c	0.0	0.2	0.0	0.0	0.5	0.0	0.0	0.0	0.7	0.0
HN ^c	0.0	0.2	0.0	0.0	1.2	0.2	0.0	0.0	0.2	0.3
Avr.	0.5	1.0	0.8	0.7	2.5	1.3	1.3	0.3	0.7	0.9
APR										
AC ^b	0.5	0.5	0.5	0.8	0.5	1.0	0.8	0.0	0.7	2.5
DC ^a	1.0	2.7	0.8	0.2	1.7	4.7	0.8	2.5	1.0	2.7
CL ^c	0.0	0.2	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0
HN ^c	0.0	0.0	0.3	0.0	0.3	0.7	0.0	0.2	0.2	0.0
Avr.	0.4	0.8	0.4	0.3	0.6	1.8	0.4	0.7	0.5	1.3
GPF										
Avr.	0.6	0.5	0.7	0.7	0.8	0.4	0.8	0.3	0.6	0.5

GPR—no. of green plants/100 placed anthers; APR—no. of green plants/100 placed anthers; GPF—no. of green plants/no. of all obtained plants. Groups followed by the same letter are not statistically significant by Tukey test ($p < 0.05$). 2,4-D—(2,4-dichlorophenoxyacetic acid). C2—(2-chloroethyl ammonium 2,4-dichlorophenoxy acetate). CD—(2-Chloroethyl trimethylammonium 2,6-dichloro-2-methoxybenzoate). TM—(Trimethylvinylammonium (2,4-dichlorophenoxy) acetate).

**Figure 4.** Boxplots of the effect of ionic acids treatment on efficiency of green plant regeneration (GPR) in analyzed genotypes in comparison to control media contains 2,4-D: (A) embryogenic cultivar AC; (B) embryogenic cultivar DC; (C) recalcitrant cultivar CL; (D) recalcitrant cultivar HN. Groups followed by the same letter are not statistically significant by Tukey test ($p < 0.05$). Middle quartile means median.

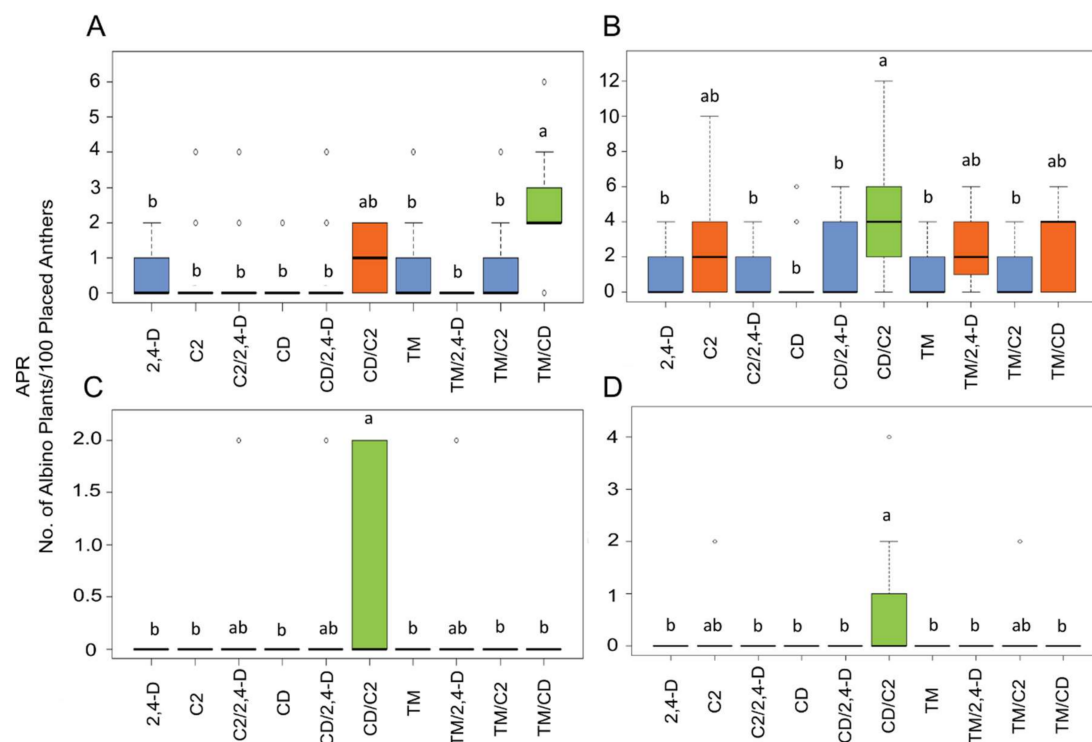


Figure 5. Boxplots of the effect of ionic liquids treatment on efficiency of albino plant regeneration (APR) in analyzed genotypes in comparison to control media contains 2,4-D: (A) embryogenic cultivar AC; (B) embryogenic cultivar DC; (C) recalcitrant cultivar CL; (D) recalcitrant cultivar HN. Groups followed by the same letter are not statistically significant by Tukey test ($p < 0.05$). Middle quartile means median.

3. Discussion

Embryogenesis of microspores in in vitro cultures is a fascinating example of plant cell totipotency. The incredible developmental plasticity of the immature male gametophyte is illustrated by the competence to change its developmental state from pollen to embryo [21]. A transition of the microspore development pathway can occur only under the influence of appropriate factors initiating this process.

Stress signaling and exogenous auxins have been suggested as inducers of ME. The most commonly used stress factors are as follows: extreme temperatures, carbon starvation, colchicine treatment, pH changes, heavy metals, high concentration of 2,4-D [22]. Treatment of the anthers with low temperatures was the stress factor applied in this experiment. The length of low temperature stress should be consistent with the methodology used and the genotypes tested. According to previous studies, Weigt et al. (2012) [23] the anthers were treated for the optimal time for the applied method, that is 7 days in 4 °C. Stress caused by low temperatures is one of the most commonly used EM inducers in many species, including wheat [5,24,25]. Not all authors classify extreme temperatures as stress. According to Djatchouk et al. [26], cold shock acts as an “anti-stress” and is rather a factor causing cytological and physiological changes that activate the cellular defense system against other stresses. Low temperature increased the accumulation of proteins characteristic for cell defense against oxidative stress and associated with active cell divisions [27].

Exogenic auxins in the induction medium are also mentioned as one of the effective factors stimulating the embryogenesis process. Anther transfer onto an induction medium without auxins causes rapid decrease in endogenous IAA [28]. Pérez-Pérez et al. [29] recommends that microspores may even be treated with compounds rising the content of endogenous auxins for effective embryogenesis. Another approach suggests supplementation of the induction medium with exogenous auxins in order to increase their overall concentration within cells. Higher demand for auxins is observed in

undifferentiated cell populations compared to specialized cells [30]. Proper levels of auxins should increase microspores plasticity and their ability to proliferate [31]. Prem et al. suggests that an auxin gradient could be the inner signal of the identity specification in ME. These phenomena are a consequence of the universal function of auxins, the most important of which are cell division, elongation, differentiation and embryonic development. In anther culture, exogenous auxins have a dual effect—they are a stress factor and simultaneously a growth regulator. Their accumulation in pro-embryo cells and a further increase of content during embryo development determine efficiency of ME. 2,4-D is one of the most common synthetic auxins used in induction media for ME of wheat and others monocot [32–34]. For this reason in our experiment the medium containing 2,4-D was used as the control. To induce ME, other authors used other synthetic auxin analogs: 1-Naphthaleneacetic acid (NAA), dicamba or picloram and its combinations [34]. In previous studies we have also used dicamba combined with 2,4-D for embryogenesis induction which increases the number of embryogenic structures and green plants of embryogenic genotypes but the anthers of recalcitrant genotypes were still characterized by very low regeneration or did not induce ME at all [35,36].

To increase the ability of wheat genotypes, especially recalcitrant ones, to form embryo and green plants, we enriched induction media by adding ILs with a similar structure to 2,4-D and dicamba. Their unique chemical and physical properties inspired us to use them in our investigation despite the fact that ILs have not been used in in vitro plant cultures thus far. In these compounds a herbicidal anion has been successfully combined with a cation exhibiting biological activity. They have a herbicidal effect and at the same time act as a growth regulator [15,19]. As ILs, they are additionally characterized by specific properties that may favorably affect in vitro plant cultures. Their low surface tension facilitates penetration into plant tissues and guarantees high biological activity [15]. Therefore, ILs dispersed in soil or foliar make it possible to reduce the doses of active substances in relation to standard synthetic auxins. Justification for their application to the induction medium also lies in their thermal stability and hydrophobic properties. They are much less volatile than 2,4-D and dicamba in *in vivo*, which allows us to predict their higher stability and more efficient exposure of cells to their action after application to the media. According to our assumptions, ILs have increased the efficiency of EM induction and plant regeneration in anther culture of wheat. All treatments increased the efficiency of average EM induction of the analyzed genotypes relative to the average efficiency of this process observed on the control medium (Table 2). A similar conclusion applies to plant regeneration, where the average for all genotypes only in one treatment did not give GPR higher than on control medium (Table 3).

In our research we noticed an interesting interaction—treatment with two auxin-like compounds had a positive effect on GP regeneration. We managed to break the genotypic recalcitrance of CL and HN varieties on 4 out of 5 media containing two hormones simultaneously. This is probably due to the complex nature of the EM process, in which four classes of proteins are responsible for auxin transport. Each of these classes has a composite structure. In *A. thaliana* only one of the domains of the ABC transporters complex (ABC B) consists of 22 ABCBs, of which 6 are associated with auxin transport [37]. In *O. sativa* there are 24 ABCBs homologs. Knockout mutants decrease polar auxin transport rates, conferring insensitivity to applied hormones. Probably, the simultaneous interaction of two regulators more effectively stimulates the polar transport of auxins to cells and/or influences genes belonging to the family of auxin response factors more intensively [37–39]. Our previous research shows that the treatment of 2,4-D and dicamba also improves the GP frequency of most genotypes analyzed in relation to the interaction of 2,4-D alone and combined with kinetin (cytokinin) [40]. We also observed a positive effect by adding 2,4-D to induction media in combination with zearelenone which has auxin-like effect [41].

The response of individual genotypes to ILs in our experiment differed significantly. The influence of genotypic dependence on EM is widely described in literature [42,43]. Moreover a significant number of wheat genotypes are characterized as recalcitrant they do not regenerate GP [35,44]. Therefore, the success of our research should be considered breaking down resistance of CL and HN varieties, which regenerated GP on medium with the addition of ILs. We observed plant regeneration

on media containing 5 out of 10 analyzed treatments. This results from the interaction of genetic factors and treatment in the medium, giving a unique combination conditioning the individual response of cultivars to applied hormones. We also observed a differentiated response to ILs in the regeneration of embryogenic genotypes, for example, from DC cultivar we obtained from 3.3 to 14.2 embryogenic structures/100 placed anthers on medium C2/2,4-D and CD/C2, respectively. These differences appear due to the complex nature of the response to EM induction and plant regeneration described above.

AP regeneration in androgenesis is an adverse phenomenon that, in addition to genotypic recalcitrance, reduces GP yield. Albinism occurs in ME-derived plants in majority of cereals (barley, oat, rice, rye, triticale and wheat). Most authors are of the opinion that AP appearance depends rather on the genotype [45]. Makowska and Oleszczuk (2014) [46] stated that albinism follows from proplastids incapable of transform into chloroplasts. Among the analyzed treatments, the most plants with chlorophyll defects regenerated on the medium with the addition of CD/C2, on which 60% of the regenerants were albinos and the least on the medium containing CD/2,4-D and TM, where GPF was 0.8. These results indicate a different effect of ILs on APR. Sibikeeva and Sibikeev (2014), [47] also imply the influence of growth regulators on the AP frequency. Our previous experimental results indicate a simultaneous effect of hormones and genotype used in the induction medium [35,36]. In this experiment, the DC variety regenerated a significantly higher number of APs compared to other genotypes.

Summing up, auxin-derived ILs enhance the efficiency of microspore embryogenesis induction and green plant regeneration in wheat. It was demonstrated that specific combinations of ILs and 2,4-D significantly stimulated the induction of microspore embryogenesis in both embryogenic and recalcitrant varieties. The results described in the paper are potentially valuable for haploids and doubled haploids obtaining and for other in vitro techniques in plants.

4. Material and Methods

4.1. Plant Material and Growing Conditions

Four spring wheat cultivars of different capacities were the research material, two of which were embryogenic: DC356/08-4-5/09 (DC), Ac Abbey (AC) and two recalcitrant: HN ROD 513,750 (HN), CLTR 7027 (CL). Three donor genotypes (DC, CL, AC) were obtained from National Small Grain Collection, United States Department of Agriculture, Agricultural Research Service Aberdeen-Idaho (USA) and one cultivar (AC) was kindly provided by Dr. Ron DePauw (Agriculture and Agri-Food Canada (AAFC), Semiarid Prairie Agriculture Research Centre, Swift Current, Canada). The donor plants were sown in two replications, in March 2017 and 2018 year in greenhouse conditions. They were grown in 20-cm pots with a soil-sand mixture (3:1). Fertilization was carried out twice, after sowing and at the beginning of stem formation using commercial fertilizer (Florovit) (Inco, Poland). There were no pesticides applied. The tillers were collected just before heading.

4.2. Donor Tillers Pre-Treatment and Sterylization

Tillers were stored in the chamber at 4 °C for seven days (light-dark cycles, 16:08 h). After pre-treatment at a low temperature, anthers isolated from the middle of spikes were crushed and the stage of nucleate of microspores was determined using a microscope. Only anthers with microspores at the mid- or late uninucleate stage were dedicated for further investigation. The spikes were surface-sterilized in a 4.85% sodium hypochlorite solution (Merck KGaA, Germany) for 4 min and rinsed three times in sterile distilled water for 5 min in a laminar flow cabinet. Then anthers were isolated from the spikes and placed on 50-mm Petri dishes (NOEX, Komorniki, Poland) containing a C17 induction medium [48].

4.3. Induction Medium Information

The C17 induction medium was modified according to Weigt et al. (2012) [23]: maltose was replaced with sucrose; the sugar concentration was 90 g L⁻¹ instead of 30 g L⁻¹; the medium was solidified by

2.5 g L⁻¹ Gelrite® (Merck KGaA, Germany) (Table S1). In addition, third-generation ILs of molecular structure similar to synthetic auxins 2,4-D and dicamba, were applied to induction media: 2-chloroethyl ammonium 2,4-dichlorophenoxy acetate [CC][2,4-D] labelled as C2; Trimethylvinylammonium (2,4-dichlorophenoxy) acetate [TMWA][2,4-D] labelled as TM; 2-Chloroethyl trimethylammonium 2,6-dichloro-2-methoxybenzoate [CC][dicamba] labelled as CD. These compounds were synthesized at Poznan University of Technology, Faculty of Chemical Technology, Poland in accordance with the methodology of References [49,50]. All ILs compounds were derivatives of herbicides with a 2,4-D (C2, TM) or dicamba (CD) anion. These salts are characterized by chemical and thermal stability. ILs exhibited higher biological activity than commonly used salts of 2,4-D and dicamba. Herbicidal ILs are nonvolatile and showed substantially lower water solubility than herbicides normally applied. All compounds used for supplementation were added to the medium in nine treatments (C2; C2/2,4-D; CD; CD/2,4-D; CD/C2; TM; TM/2,4-D; TM/C2; TM/CD) aiming for a total concentration of 2 mL/L (Table 4). The medium containing only 2,4-D was used as a control. They were added to the cooled medium after dissolving in water and filtering with a 0.22 µm MF-Millipore™ filter (Merck Millipore™, Germany).

Table 4. Ionic liquids (ILs) and 2,4-D combinations in the C17 medium used for anthers treatment.

Treatment	Content of Ionic Liquids in the Induction Medium (mL/L)			
	2,4-D	C2	CD	TM
2,4-D (Control)	2	-	-	-
C2	-	2	-	-
C2/2,4-D	1	1	-	-
CD	-	-	2	-
CD/2,4-D	1	-	1	-
CD/C2	-	1	1	-
TM	-	-	-	2
TM/2,4-D	1	-	-	1
TM/C2	-	1	-	1
TM/CD	-	-	1	1

2,4-D—(2,4-dichlorophenoxyacetic acid). C2—(2-chloroethyl ammonium 2,4-dichlorophenoxy acetate). CD—(2-Chloroethyl trimethylammonium 2,6-dichloro-2-methoxybenzoate). TM—(Trimethylvinylammonium (2,4-dichlorophenoxy) acetate).

4.4. Course of Anther Culture

On each Petri dish there were 50 anthers; each experimental combination was repeated 6 times/year. The dishes were stored in darkness at a temperature of 28 °C for 6–8 weeks. In the meantime, rate of embryogenic structures (ES) development of was observed. For this purpose the time from the beginning of the culture to the appearance of ES with a diameter of 0.5 cm was estimated. ES were counted and replaced on the MS regeneration medium [51] where 0.5 mg L⁻¹ of NAA and kinetin was added. The medium was solidified with agar (Merck KGaA, Germany) concentrated at 0.6% and stored in the chamber at a temperature of 24 °C and a 16/8-h (light/dark) photoperiod. The plants' regeneration was observed after 2–4 weeks.

4.5. Data Analysis

The following parameters were monitored: ESF—the efficiency of embryogenic structure formation (the number of embryogenic structures, ES, per 100 plated anthers); GPR—the efficiency of green plant regeneration (defined as the number of green plant, GP, per 100 plated anthers); APR—the efficiency of albino plant regeneration (defined as the number of albino plant, AP, per 100 plated anthers); GPF—green plant frequency (defined as the number of green plants per all obtained plants).

Statistical analysis of the experiment began with the assessment of three factors: year, treatment and genotype, using ANOVA with Tukey's HSD post-hoc test. Statistical analyses were performed

separately for ES, GP and AP. The results were presented in the form of boxplots with groups of similar objects. Then, Principal Component Analysis (PCA) was used to assess the influence of studied ILs in the induction medium on the ESF, GPR and APR and also the relationship between observed variables. The result of PCA was presented as a biplot. The R software, version 3.6.2 [52], was used for statistical calculations.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/6/839/s1>, Table S1. C17 induction media composition with modifications.

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