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Brief Report

Evaluation of In Vitro Morphogenic Response of *Triticum urartu*, a Donor of A^u Genome of Modern Wheat

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Abstract: *Triticum urartu* Thum. ex Gandil. is a wild diploid wheat species (2n = 2x = 14) that is an A^u genome donor of modern polyploid cultivars of durum and bread wheat. In the last decade, this relict species has attracted breeders as donors of various agronomically important characteristics to broaden the genetic diversity of cultivated wheat. In addition, *T. urartu* can be considered as a model species for studying the evolution, biology and genomics of wheat without the cross-influence of homologous sub-genomes. Various genetic engineering technologies, including transgenesis and genome editing, may be applied to facilitate the functional characterization of genes located in A chromosomes. Such biotechnological techniques are still required for the efficient tissue culture systems to allow easy plant regeneration. The objective of our study was to assess the abilities of in vitro plant regeneration from zygotic immature embryo-derived tissues of spring and winter types of *T. urartu*. Three synthetic auxins, 2,4-D, Dicamba and Picloram, at four concentrations were studied to stimulate morphogenic responses in spring *T. urartu*. The induction medium supplemented with 4 mg·L^{-1} Dicamba stimulated the highest frequency of regenerable callus production (65.8%), promoting the generation of 5.7 plants. Although the presence of 2 mg·L^{-1} 2,4-D was less effective in stimulating regenerable callus formation (53.2%) than Dicamba, it allowed the regeneration of more plants from one regenerable callus (9.3 plants). These two treatments also successfully initiated morphogenesis in winter assertions; however, their regenerative capacity was generally lower. The frequency of regenerable callus production was accession-dependent and fluctuated within 31.3 to 49.2%, with a formation of an average 2.2–5.8 plants per callus. The relatively simple and fast regeneration system described in this study could be further used as the basis for regenerating transgenic plants of *T. urartu*.

Keywords: diploid wheat; immature embryo; callusogenesis; auxins; plant regeneration

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1. Introduction

The *Triticum* genus includes numerous wild, domesticated and synthetic species carrying various combinations of subgenomes, such as A^u, A^m, B, D and G. Two interrelated genomes, A^u and A^m, are wheat-derived genomes, while the others, B, D, and G, are considered to be derived by diploid species of the genus *Aegilops* [1,2]. Modern wheat cultivars are generally referred to as two species: hexaploid bread wheat (*T. aestivum*, 2n = 6x = 42), consisting of A^uA^uBBDD subgenomes, and tetraploid durum wheat (*T. durum*, 2n = 4x = 28), consisting of A^uA^uBB subgenomes. The polyploid nature of modern wheat complicates the determination of functions of homologous genes derived from A, B, and D subgenomes and makes the elucidation of their individual influence on certain agronomic- and yield-related traits problematic. Since the A genome is considered to be a basic genome of wheat, *T. urartu*, as a progenitor of durum and bread wheat, is of great importance for a comprehensive study of the genomics and phylogeny of the genus *Triticum* [1,3].

The genome size of the diploid *T. urartu* is only about 4.94 Gb, much smaller compared to the 17 Gb genome size of bread wheat. *T. urartu* resembles cultivated wheats in its biology and, in contrast to another A-genome species *T. monococcum* L., it can be crossed with durum and bread wheat (*T. eastivum* L.) [4]. Recently, the genome of *T. urartu* was sequenced [5], providing new insights into the A genome functional activity and the evolutionary process of *Triticeae* [6]. For this reason, *T. urartu*, carrying the reference A^u subgenome, can be used as a model species containing all the main genetic and molecular data to streamline agronomic improvements in modern cultivated wheat.

T. urartu was first discovered in 1934 in Armenia [7], but being a two-grain cereal species, it was not botanically separated from einkorn wheat until the end of the 20th century [8]. As a relict species, *T. urartu* is an endemic cereal distributed in various areas, including the eastern Mediterranean coast, Mesopotamia and Transcaucasia [6]. Recent genetic studies have revealed that in contrast to the A genome of the current wheat cultivars, the natural populations of *T. urartu* possesses a higher level of allelic diversity accumulated over time [9]. Since *T. urartu* carries many adaptive and agronomically important traits, including being highly photosynthetic and disease-resistant [10–12], it can be useful for breeding new lines and modern varieties with an expanded genetic base using conventional breeding methods and modern agro biotechnological genetic engineering approaches.

Modern methods of plant biotechnology, such as genetic engineering and genome editing, can contribute to the exploration of *T. urartu* as a model species, helping to isolate and study the functional activity of relevant genes when the influence of other homologous subgenomes is eliminated. *T. urartu* is still not involved in such research, while the other diploid species, such as einkorn wheat, has already been successfully genetically engineered [13,14]. To date, nearly fifteen wild and cultivated *Triticum* species have already been studied for somatic embryogenesis and plant regeneration [15–18]. Although most of the experiments were carried out using varieties of durum and bread wheat [16,19–24], it was also shown that various tetraploid and hexaploid species carrying A^U, B and D subgenomes are able to generate plants in vitro with varying degrees of efficiency, while the species *T. dicoccum* (Schrank) Schubl. (A^uB genome), *T. polonicum* L. (A^uB genome) and *T. spelta* L. (A^uBD genome) have a high regenerative capacity [16–18]. Contrasting to other species, the tissue culture of wheat species with G subgenome composition, such as *T. timopheevii* Zhuk. (A^mG) and *T. kiharae* Dorof. et Migusch (A^mBG), displayed low morphogenesis with a high fraction of albino plants among regenerated plants [25,26]. Among the studied diploid wheat species, *T. sinskajae* A.Filat. et Kurk (A^m genome) was reported to display effective somatic embryogenesis and plant regeneration in vitro [17], while the einkorn wheat (*T. monococcum* L., A^m genome) displayed a low ability to regenerate plants, and only a significant enrichment of induction media [13,15] or the transgenic manipulations could increase the morphogenic response of einkorn wheat [14]. Tissues of *T. urartu* are almost unexplored in terms of their ability to regenerate plants in vitro [15], while efficient plant tissue culture is still an important part of the current protocols for wheat genetic transformation and gene editing. The general consensus from research involving tissue culture of *Triticum* spp. is that the isolation and cultivation of immature embryos is still the best approach to induce a morphogenic response, while the application of mature embryo-derived tissues is significantly less efficient and can be applied for only a restricted number of germplasms [17,27–29].

Here, we attempted to study the plant regeneration responses of immature zygotic embryos of *T. urartu*. In the present study, four concentrations of three auxins were supplemented into the callus induction medium to analyze the morphogenic callus production and plant regeneration. The two better variants were then used to study winter types of *T. uratu* for their abilities to generate plants in vitro. Our results are the first step that will facilitate the further use of *T. urartu* as a natural genetic model for wheat molecular breeding using transgenic and genome editing technologies.

2. Materials and Methods

2.1. Plant Material and Donor Plant Growth

The plant materials used in this study were four accessions of the *T. urartu* Thum. ex Gandil., including one spring (PI428197) and three winter (K-33869, K-62459, PI538736) accessions from the collection of Prof. N.P.Goncharov (Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia) and Vavilov Institute of Plant Genetic Resources (VIR, Sankt-Peterburg, Russia). Plants used for isolation of immature zygotic embryos were grown in boxes in a glasshouse under temperature-controlled conditions (18–25 °C) with supplementary light during the winter (up to 200 μmol/m^{-2} s^{-1}) providing a 16 h photoperiod. The winter-type plants were vernalized after the appearance of a fourth leaf for 60 days at 4–8 °C in a cold greenhouse under a 12 h day length photoperiod. The intact immature zygotic embryos of about 1 to 1.5 mm in size were used as explants in all experiments. Embryos were freshly isolated from young caryopses (8–10 days post-anthesis stage) using the protocol described previously [28].

2.2. Culture Media and Culture Conditions

The culture media were based on Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose and solidified with 7 g L^{-1} agar (European type technical grade, Panreac, Spain), and the pH was adjusted to 5.8–5.9 prior to autoclaving. The medium for induction of the callus was supplemented with 150 mg·L^{-1} asparagine and various auxins. To induce callusing, freshly isolated embryos were placed scutellum face up in 100 mm × 20 mm glass Petri dishes containing 25 mL of medium and cultured for 30 days in the dark at 25 ± 1 °C. After that, all explant-produced calli were transferred into glass jars (8–10 calli per flasks) containing 100 mL of phytohormone-free MS medium to induce plant regeneration and rooting. Flasks were maintained for 30 days in the culture room at 24 ± 2 °C under artificial light (100 μmol m^{-2} s^{-1} provided by Philips cool white and OSRAM fluora fluorescent lamps) with a daily photoperiod of a 16/8 h light/dark cycle.

2.3. Effect of Auxins

Three types of auxins, namely 2,4-D, Dicamba and Picloram, were used to study morphogenic callus induction. Four concentrations of each auxin (2, 3, 4 or 5 mg·L^{-1}) were tested. Two Petri dishes with 15–20 immature zygotic embryos were used as a replicate for each concentration treatment, and there were four or five replicates for the experiment. Experiment was performed using a spring accession PI428197 of the *T. urartu*.

2.4. Initiation of Embryogenic Callus in Winter T. urartu

Immature zygotic embryos of three winter-type accessions of the *T. urartu* were studied to induce morphogenic cultures using two hormonal treatments. Then, 2 mg·L^{-1} 2,4-D or 4 mg·L^{-1} Dicamba were added to the callus-induction medium for the evaluation of accessions. Two Petri dishes, each containing 20–22 explants, were used as a replicate for each accession × auxin variant. Six independent and identical experiments were performed.

2.5. Statistical Analysis

Data were collected on a per-plate/jar basis. The percentage of morphogenic callus induction and regenerating callus production was calculated per initial number of cultured zygotic embryos. Morphogenic calli producing at least one green plantlet longer than 1 cm were classified as regenerating calli. The regeneration coefficient was calculated as an average number of regenerated green plantlets per initially cultured immature embryo. Within the experiments, data were analyzed by one-way or two-way ANOVA using Statistica10 software (©StatSoft Inc., Tulsa, OK, USA), and the mean separation was carried out using Duncan's post hoc test at $p \leq 0.05$.

3. Results

3.1. Effect of Auxins

3.1.1. The Effect of 2,4-D Concentrations

All the tested 2,4-D concentrations successfully induced the formation of calli in cultured immature embryos of *T. urartu* with a frequency of 96.5–99.7% (Table 1). Generally, calli are easily formed during the first weeks of culture, while the formation of morphogenic structures in callusing explants was started after 2 weeks of cultivation. Morphogenic calli represented nonfriable yellow-white nodular embryogenic structures and generally covered half of the callus surface. They were easily distinguished from the friable translucent non-regenerative calli at the end of cultivation (Figure 1A). At the four tested levels of auxin, the higher portion of explants producing embryogenic structures (63.3%) was observed on the medium supplemented with 2 mg·L^{-1} 2,4-D (Table 1). Higher concentrations of auxin (4 or 5 mg·L^{-1} 2,4-D) were less effective as the efficiency of morphogenic callus formation decreased to 51.8–47.7%.

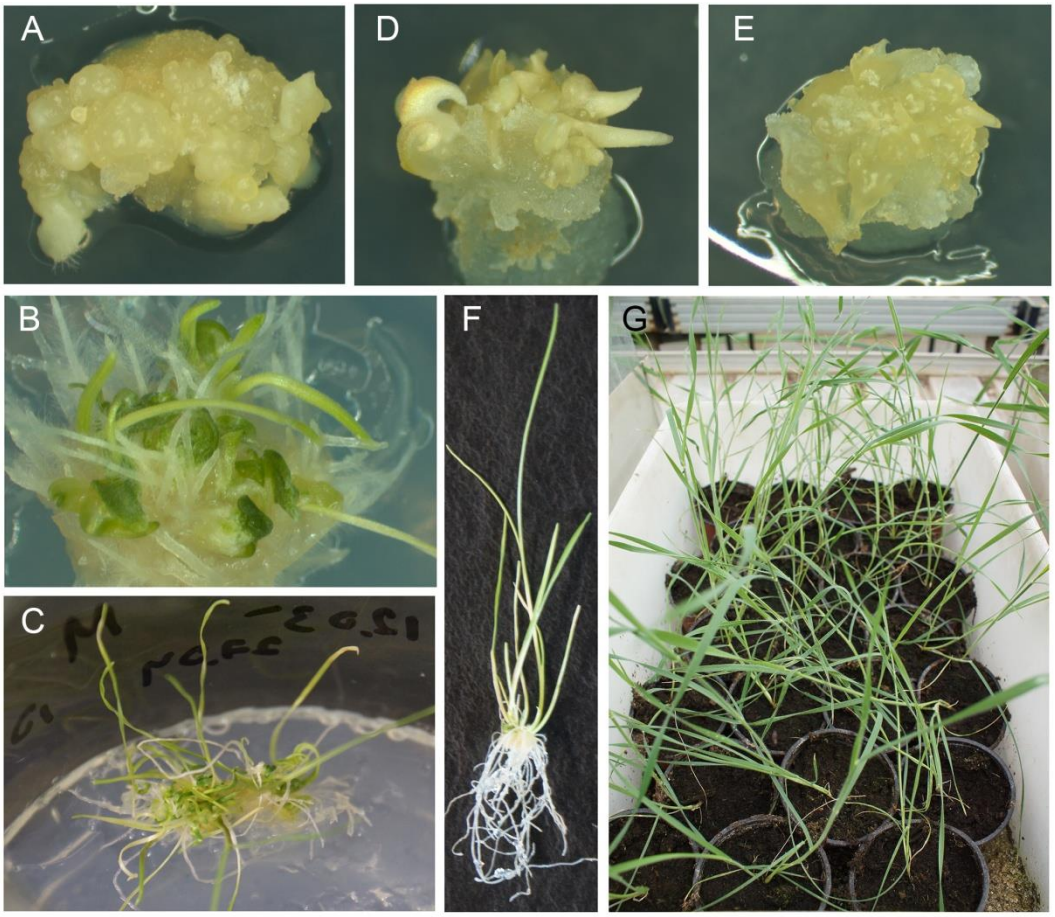

Figure 1. Plant regeneration process in immature embryo-derived culture of *Triticum urartu*, spring accession PI428197. The appearance of embryogenic calli with somatic embryos on the medium supplemented with 2 mg·L^{-1} 2,4-D (**A**) after 30 days of culture in the dark; the regenerable calli with green buds and leafy structures on the phytohormone-free medium (**B**), after 10 days of culture in the light; formation of plantlets from regenerable calli (**C**), after 20 days in the light; embryogenic callus formation from immature embryo after induction with 4 mg·L^{-1} Dicamba (**D**) and 4 mg·L^{-1} Picloram (**E**) after 30 days of culture in the dark; healthy plantlets with roots before being transferred to greenhouse (**F**); regenerated plants grown under greenhouse conditions (**G**).

Table 1. The effect of 2.4-D concentrations on morphogenic callus production and plant regeneration in immature embryo-derived cultures of *Triticum urartu*, spring accession PI428197.

Concentration (mg·L^{-1})	Callus Induction (%)	Morphogenic Callus Formation (%)	Percentage of Regenerating Calli (%)	No. of Plantlets per Regenerable Calli	Regeneration Coefficient *
2	99.7 ns	63.3 b	53.2 ns	9.3 ns	5.1 b
3	98.6 ns	58.3 ab	43.9 ns	8.6 ns	3.9 ab
4	99.2 ns	47.7 a	39.54 ns	8.8 ns	3.6 ab
5	96.5 ns	51.8 ab	42.6 ns	6.9 ns	2.9 a

* average number of plants regenerated per single immature zygotic embryo. Calli were initiated from embryos within 30 days on callus-induction medium with the subsequent subcultivation on a medium without phytohormones. Means with the same letter in the column had no significant differences according to Duncan's multiple range test ($p < 0.05$), ns = non significant.

After transferring to hormone-free medium, morphogenic calli formed green sectors and buds, which further established into bipolar structures and developed into visible plantlets within 7 to 10 days of cultivation under light (Figure 1B). The plant regeneration capacity of 2,4-D-induced calli fluctuated from 34.4% to 53.2%. Despite the fact that statistical analysis revealed that auxin concentrations had no significant effects on this parameter, there was a tendency to form more regenerable calli when the immature embryos were cultivated using lower concentrations of 2.4-D (Table 1). Most of the regenerating calli produced 7–12 shoots (Figure 1C) and no clear influence of auxin dosage was proved. At the end of the cultivation on the hormone-free medium, regenerated shoots developed prominent roots (Figure 1F), easily transplanted into the pots and successfully survived under greenhouse conditions (Figure 1G). The overall efficiency of plant regeneration from initially cultivated explants of *T. urartu* was affected by 2.4-D concentration (Table 1). An increase in 2.4-D level in callus induction medium generally leads to a decrease in the number of regenerated plants per cultivated immature embryo. Thus, the highest number of regenerated plants (5.2) was observed at 2 mg·L^{-1} 2,4-D.

3.1.2. The Effect of Dicamba Concentrations

Similar to the previous experiment, no significant differences in callus induction were observed when media were supplemented with different Dicamba concentrations. Almost all the cultivated embryos of *T. urartu* produced calli with a frequency of 99–100% (Table 2). The appearance of primary calli was observed at 4–7 days after culture initiation through the swelling of scutella's margins. In contrast to calli induced by 2,4-D, the calli developed in the presence of Dicamba were more compact; morphogenic calli developed further were harder, bright yellow-white and non-translucent with a smaller number of nodular structures (Figure 1D). Developed nodular calli tended to convert into leafy structures towards the end of the cultivation on the callus induction medium (Figure 1D).

Table 2. The effect of Dicamba concentrations on morphogenic callus production and plant regeneration in immature embryo-derived cultures of *Triticum urartu*, spring accession PI428197.

Concentration (mg·L^{-1})	Callus Induction (%)	Embryogenic Callus Formation (%)	Percentage of Regenerating Calli (%)	No. of Plantlets per Regenerable Calli	Regeneration Coefficient *
2	100.0 ns	69.1 a	39.9 a	4.0 a	1.3 a
3	100.0 ns	75.5 ab	59.0 ab	4.6 ab	2.7 b
4	100.0 ns	79.4 ab	65.8 b	5.7 ab	4.0 c
5	99.0 ns	81.0 b	59.9 ab	5.9 b	3.4 bc

* average number of plants regenerated per single immature zygotic embryo. Calli were initiated from embryos within 30 days on callus induction medium with the subsequent subcultivation on a medium without phytohormones. Means with the same letter in the column had no significant differences according to Duncan's multiple range test ($p < 0.05$), ns = non significant.

In general, Dicamba stimulated the formation of morphogenic callus (69–81%) better than 2,4-D (48–63%). Media supplemented with 4–5 mg·L^{-1} of Dicamba were more effective in promoting both morphogenic and regenerable callus production compared to lower

concentrations, especially 2 mg·L^{-1} of auxin (Table 2). At a Dicamba concentration of 4 mg·L^{-1}, an average of 65.8% of cultured explants were able to regenerate plants. This was the highest level among the three tested auxins. The induction by Dicamba of compact morphogenic calli that show earlier conversion into leafy structures resulted in a lower number of regenerated plantlets compared to 2,4-D. Among analyzed concentrations, the incorporation of 4–5 mg·L^{-1} Dicamba into callus-induction medium demonstrated a better promoting effect, resulting in the formation of 5.7–5.9 plants per regenerating callus. At the same time, at a better 2,4-D concentration, the number of regenerated plants was higher and achieved 9.3 plants per calli (Table 1). For this reason, the overall in vitro efficiency of plant regeneration per initial zygotic embryo was somewhat lower upon application of Dicamba. The highest regeneration coefficient (4.0 plants per isolated embryo) was scored at 4 mg·L^{-1} Dicamba. The unpaired t-test found no significant differences ($p = 0.31$) between two variants of auxins (4 mg·L^{-1} Dicamba vs. 2 mg·L^{-1} of 2,4-D) that displayed the best achievements in this parameter (4.0 vs. 5.1 plants per one isolated embryo, respectively).

3.1.3. The Effect of Picloram Concentrations

The difference between the media containing various concentrations of Picloram was insignificant for callus initiation from cultured zygotic embryos of *T. urartu* (Table 3), as an identically high ratio of callus formation (99–100.0%) was observed in all the variants. In contrast to Dicamba, morphogenic calli appearing during cultivation in Picloram-enriched medium mostly consisted of translucent off-white proliferative bulges and soft nodular structures. Visually, the total amount of morphogenic callus produced by explants was smaller (moderate, greater) than on medium supplemented with 2,4-D (Figure 1A,E), but the rate of morphogenic structure formation was similar between two auxins, fluctuating in Picloram from 45.9% (2 mg·L^{-1}) to 65.4% (4 mg·L^{-1}). From the four tested levels of Picloram, the concentration of 2 mg·L^{-1} was the most unsuccessful in the induction of regenerable calli and plant regeneration (Table 3), demonstrating the lowest ability to initiate in vitro tissue culture. Higher concentrations, 3, 4 and 5 mg·L^{-1}, were almost equal in all measured parameters, including the efficacy of morphogenic callus production (59.6%, 65.4 and 59.0), the rate of regenerable calli (55.4%, 57.5% and 50.9%) and average number of regenerated plantlets (4.9, 5.1 and 4.8, per regenerable calli). As a result, there were no significant differences in regeneration coefficients when a range of 3 to 5 mg·L^{-1} of Picloram was used; the average numbers of regenerated plants per each initially cultured explant were 2.8, 3.0 and 2.7, respectively (Table 3). In general, Picloram-containing media were less effective for plant regeneration than the cultivation on 2 mg·L^{-1} of 2,4-D (the best variant for 2,4-D). Statistical analysis indicated that the regeneration coefficient of 3.0 plants per initial immature embryo resulted after the application of 4 mg·L^{-1} Picloram (a slightly better variant compared to the others) was significantly lower at a high confidence level of 99% ($p = 0.0084$) compared to 5.2 plants observed at 2 mg·L^{-1} of 2,4-D. The difference with the best Dicamba treatment of 4 mg·L^{-1} (4.0 plants per initial immature embryo) cannot be proved as the threshold of confidence ($p = 0.092$) was higher than the 95% confidence level ($p < 0.05$).

Table 3. The effect of Picloram concentrations on morphogenic callus production and plant regeneration in immature embryo-derived cultures of *Triticum urartu*, spring accession PI428197.

Concentration (mg·L^{-1})	Callus Induction (%)	Morphogenic Callus Formation (%)	Percentage of Regenerating Calli (%)	No. of Plantlets per Regenerable Calli	Regeneration Coefficient *
2	98.9 ns	45.9 a	30.6 a	2.9 a	0.9 a
3	98.9 ns	59.6 b	55.4 ab	4.9 b	2.8 b
4	98.9 ns	65.4 b	57.5 b	5.1 b	3.0 b
5	100.0 ns	59.0 b	50.9 b	4.8 b	2.7 b

* average number of plants regenerated per single immature zygotic embryo. Calli were initiated from embryos within 30 days on callus induction medium with the subsequent subcultivation on a medium without phytohormones. Means with the same letter in the column had no significant differences according to Duncan's multiple range test ($p < 0.05$), ns = non significant.

3.2. Initiation of Morphogenic Response in Winter Type of T. urartu

In the next experiment, we analyzed in vitro tissue responses of three winter type of *T. urartu*. Like in the previous experiment, the same two-step protocol was used to induce morphogenesis. It included 30 days of cultivation of immature zygotic embryos in the dark on callus-induction media following 30 days of plant differentiation in the light on media without phytohormones. In a previous study, explants of spring-type *T. urartu* showed that supplementation of 4 mg·L^{-1} Dicamba or 2 mg·L^{-1} 2,4-D in callus-induction medium gives statistically similar results in terms of the regeneration coefficient. Dicamba was more effective in the stimulation of morphogenic calli, while regenerable calli induced in the media supplemented with 2,4-D generated more plantlets. Knowing that various germplasms of the same species can differentially react on the same hormonal composition of tissue culture medium, we used both of the indicated variants to analyze the morphogenic response of winter *T. urartu* accessions. The results of the experiment are shown in Figure 2.

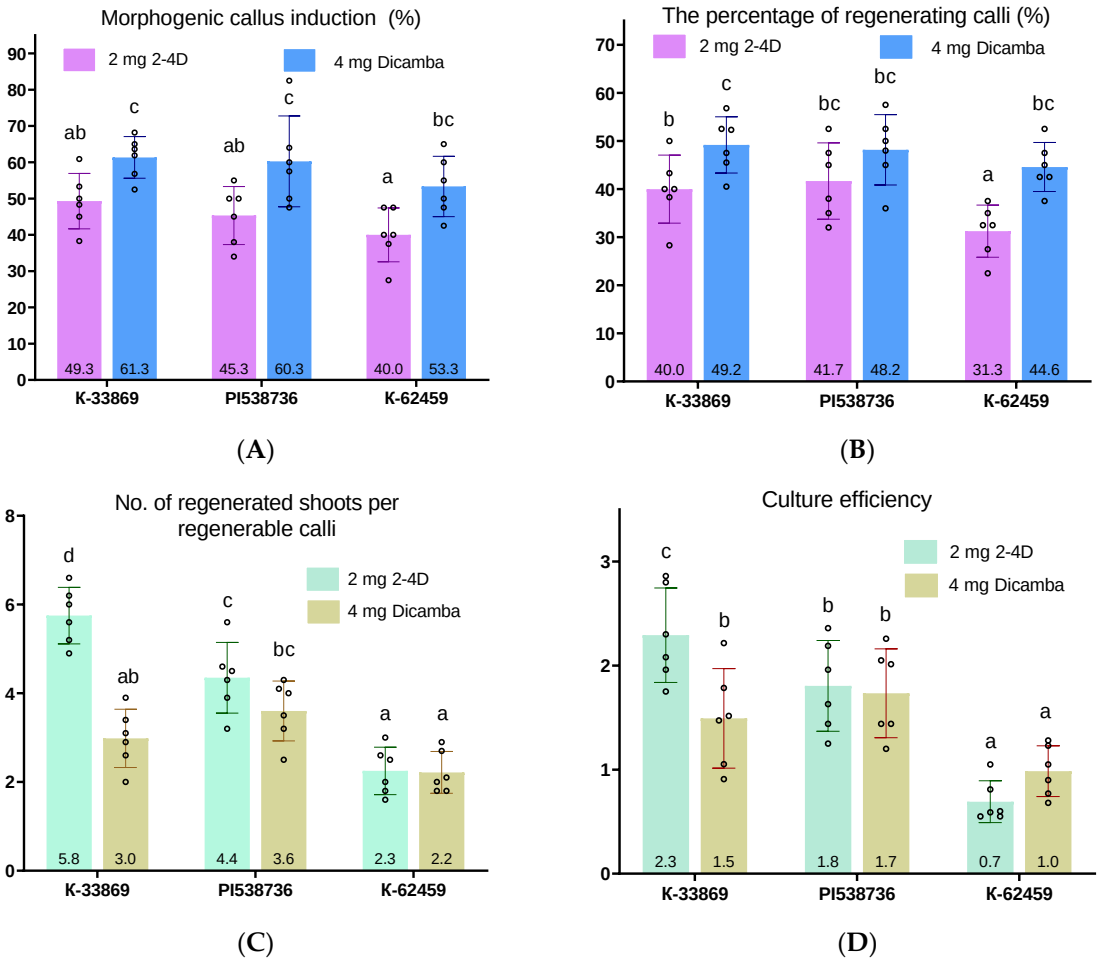

Figure 2. Morphogenic response of three winter accessions of *Triticum urartu* cultivated on two callus-induction media: the first was supplemented with 2 mg·L^{-1} 2,4-D; the second was supplemented with 4 mg·L^{-1} 2,4-Dicamba. (**A**) The percentage of explants that produced morphogenic calli (%); (**B**) the percentage of explants that produced regenerable calli (%); (**C**) number of plantlets regenerated from one regenerating calli (%); (**D**) tissue culture efficiency, calculated as the average number of plants regenerated per single isolated immature embryo. Data collected from two media were subjected to two-way ANOVA; means with the same letter in the column had no significant differences according to Duncan's multiple range tests ($p < 0.05$).

Cultivated immature zygotic embryos of winter *T. urartu* successfully produced calli with a frequency of 100% on both auxin-supplemented media. The presence of 2 mg·L^{-1}

2,4-D in callus-induction medium stimulated the formation of morphogenic structures within a range of 40.0–49.3% (Figure 2A). In the case of Dicamba, a higher mean ratio of morphogenic callus production was observed, as more than 50% of cultivated zygotic embryos showed formation of morphogenic structures (Figure 2A). Similarly, all accessions showed a better ability to develop regenerable calli after cultivation on medium containing Dicamba. Almost half of the explants of K-33869 (49.2%) and PI538736 (48.2%) cultured in the presence of Dicamba were able to produce regenerating calli, while 2,4-D-contaning medium induced regenerable structures at the frequencies of 40.0% and 41.7%, respectively. K-62459 produced fewer regenerable calli but, like the other two winter germplasms, also responded more positively to the presence of Dicamba in the induction medium (44.6%) than to the addition of 2,4-D (31.3%). Two-way ANOVA confirmed the auxin-dependent effect for both morphogenic and regenerable callus production at $p < 0.0001$, while the influence of the accessions was proved only for the morphogenic callus rate ($p = 0.021$) at a 95% confidence level.

The type of auxin supplemented to the induction media had a significant effect on the plant regeneration in K-33869 (Figure 2C). A greater mean number of regenerants (5.8 plants per regenerable callus) resulted from calli of K-33869 developed on the medium containing 2 mg·L^{-1} 2,4-D. Although PI538736 also showed a tendency to form more plants after induction with 2,4-D (4.4 per regenerable callus) than after Dicamba treatment (3.6 per regenerable callus), the post hoc analysis revealed that the difference is not critical ($p = 0.75$). Among winter accessions of *T. urartu*, calli of K-62459 showed the lowest ability to produce green plants, and auxin had no effect on this parameter, as the same mean number of regenerated plants (2.0–2.2 per regenerable callus) was observed using both tested media.

Two-way ANOVA analysis showed that tissue culture efficiency was more significantly influenced by the accession ($p < 0.0000$) and the accession x auxin interaction ($p = 0.0058$) than by the auxin type ($p = 0.1474$). The choice of auxin for supplementation into the induction medium had no significant effect on this parameter in two tested accessions: K-62459 and PI538736 (Figure 2D). Induced calli of K-62459 showed the lowest tissue culture efficiency, producing almost one plant per each initially cultured zygotic embryo using both induction media. PI538736 calli scored 1.7 and 1.8 plants per explant when Dicamba and 2,4-D, respectively, were added to the induction medium (Figure 2D). K-33869 showed a highest in vitro culture efficiency of zygotic embryos among the tested winter accessions only in the case of 2,4-D supplementation into the callus induction medium (2.3 plants per initial explant), while cultivation on Dicamba-containing medium was equal to the PI538736 regeneration coefficient (1.5).

4. Discussion

The acquisition of the morphogenic potential in cultures of wheat and close relative cereal species is mostly initiated through the primary and secondary somatic embryogenesis when competent cells within a zygotic embryo respond to signals such as endogenous auxin and then de-differentiate into regenerable structures [30]. For a wide range of polyploid durum and bread wheat cultivars, the treatment with 1.5–2 mg·L^{-1} of 2,4-D is usually sufficient to induce somatic embryogenesis at a satisfactory level [16,19,20]. Media containing 2,4-D were also reported to be sufficient to induce morphogenic calli in various wild and cultivated diploid, tetraploid and hexaploid wheat species [17,18]. In the present study, immature zygotic embryos of diploid *T. urartu* accessions positively reacted to the presence of 2,4-D in the callus-induction medium as one-third to one-half of cultured explants produced regenerable calli. Since genotype dependency is still a major obstacle in inducing efficient morphogenesis, other substances such as Picloram or Dicamba were involved as equal or even better alternative auxins. Picloram used as a sole auxin positively influenced morphogenesis in some cultivated wheat germplasms [21,22,25]. In the present study, the incorporation of Picloram (3–5 mg·L^{-1}) into the callus-induction medium was more effective for regenerable callus induction than 2,4-D, but contrasting to 2,4-D, it promoted a lower number of regenerated plants.

Dicamba was reported to be more effective than other auxins in inducing morphogenesis in diploid einkorn [13] and polyploid wheat cultivars [23]. In durum wheat, it was shown that replacing 2,4-D with Dicamba enhanced *Agrobacterium*-mediated T-DNA transfer into regenerable cells and provided better transgenic plant development, though all analyzed genotypes were able to produce morphogenic calli using both auxins [28]. Similarly, Dicamba significantly increased the plant regeneration from the immature embryos of bread wheat inoculated with *Agrobacterium* [23]. In our study, the addition of Dicamba to the medium also better stimulated morphogenic callus formation than 2,4-D and Picloram; however, higher concentrations (3–5 mg·L^{-1}) were required to achieve certain results. On the other hand, the mean number of regenerated shoots due to added auxins was significantly decreased in two *T. urartu* accessions when Dicamba was supplemented into a callus-induction medium. To overcome such individual auxin shortcomings, it was proposed to firstly cultivate explants on a medium containing Dicamba (to increase the rate of morphogenic callus induction) and then transferred induced structures to a fresh medium containing other auxin types to increase the number of regenerated plants. This strategy has been successfully used for a range of bread wheat and triticale cultivars [31,32]. Another approach is to simultaneously enrich the induction medium with two different auxins. A combination of 2,4-D with Picloram in callus-induction medium is frequently used to produce transgenic bread wheat plants after *Agrobacterium*-mediated transfer of T-DNA [33–35]. Another combination, namely 2,4 and Dicamba, was also reported to successfully produce transgenic wheat plants [36]. Taking into account the results of our study, such approaches hold key information for increasing morphogenesis in *T. urartu* using auxin combinations, but combinatory effects should be accurately tested as some negative results of adding together two auxins to the medium, such as a reduction in plant regeneration, were also reported [37].

In the current study, we used a simple two-step protocol that included the development of embryogenic/organogenic structures on the callus-induction medium enriched with one of the auxins and then the induction of plant regeneration from the produced structures on the phytohormone-free medium. Such an approach allowed generating an average of 3.0–5.1 plants per initial explant in spring *T. urartu* and up to 2.3-plants in winter accessions. Previously, Eudes et al. [15] showed the possibility of generating 1.7 plants from the scutellum of the spring *T. urartu* line 17111. To date, this report was the first and only research using *T. urartu* as a cereal species for tissue culture evaluations. In contrast to our study, in the published report, a small number of explants with only two replications were studied due to contamination of isolated zygotic embryos. Our protocol allowed us to initiate tissue culture of *T. urartu* without contamination problems and proved to initiate more plants per initial explant, especially when 2,4-D is used as an endogenous inductor of callogenesis. Unfortunately, the data of Eudes et al. [15] did not include the information concerning the rate of embryogenic/morphogenic induction and still have not been reproduced by other authors. Moreover, a multi-stage protocol consisting of five culture media (direct somatic embryogenesis, secondary somatic embryogenesis, germination, regeneration and rooting media) containing a significantly larger number of components (six carbohydrates, eighteen amino acids and five plant growth regulators) was required to stimulate plant regeneration in *T. urartu* [15], unlike the simpler two-step protocol described here.

Obviously, the morphogenic efficiency of immature embryo-derived tissues of *T. uaratu* is lower than that of the well-regenerating cultivars of bread wheat that are actively used in genetic transformation and genome editing research such as 'Bobwhite', 'Fielder' or 'Chinese Spring' [29]. However, it should be considered that *T. urartu*, as a wild diploid species, has rather small grains and significantly smaller embryos than polyploid wheat species, especially at the isolation stage, which may affect the overall effectiveness of the tissue culture. Nevertheless, we believe that the morphogenic reactions achieved in this study are sufficient to conduct further experiments on *T. uaratu* using modern

biotechnologies such as transgenesis and genome editing to expand the molecular and genetic knowledge necessary for the development of new innovative wheat varieties.

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Data Availability Statement: The data generated or analyzed during this study are included in this published article. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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References

1. Dvorák, J.; Terlizzi, P.; Zhang, H.B.; Resta, P. The evolution of polyploid wheats: Identification of the A genome donor species. *Genome* **1993**, *36*, 21–31. [CrossRef] [PubMed]
2. Goncharov, N.P. Genus *Triticum* L. taxonomy: The present and the future. *Plant Syst. Evol.* **2011**, *295*, 1–11. [CrossRef]
3. Peng, J.H.; Sun, D.; Nevo, E. Domestication evolution, genetics and genomics in wheat. *Mol. Breeding* **2011**, *28*, 281–301. [CrossRef]
4. Grewal, S.; Hubbart-Edwards, S.; Yang, C.; Scholefield, D.; Ashling, S.; Burridge, A.; Wilkinson, P.A.; King, I.P.; King, J. Detection of *T. urartu* Introgressions in Wheat and Development of a Panel of Interspecific Introgression Lines. *Front. Plant. Sci.* **2018**, *9*, 1565. [CrossRef] [PubMed]
5. Ling, H.-Q.; Ma, B.; Shi, X.; Liu, H.; Dong, L.; Sun, H.; Cao, Y.; Gao, Q.; Zheng, S.; Li, Y.; et al. Genome sequence of the progenitor of wheat A subgenome *Triticum urartu*. *Nature* **2018**, *557*, 424–428. [CrossRef]
6. Wang, X.; Hu, Y.; He, W.; Yu, K.; Zhang, C.; Li, Y.; Yang, W.; Sun, J.; Li, X.; Zheng, F.; et al. Whole-genome resequencing of the wheat A subgenome progenitor *Triticum urartu* provides insights into its demographic history and geographic adaptation. *Plant. Commun.* **2022**, *3*, 100345. [CrossRef]
7. Dorofeev, V.F. The variability and breeding value of Armenian wheats. *Euphytica* **1968**, *17*, 451–461. [CrossRef]
8. Heun, M.; Haldorsen, S.; Vollan, K. Reassessing domestication events in the Near East: Einkorn and *Triticum urartu*. *Genome* **2008**, *51*, 444–451. [CrossRef]
9. Wang, X.; Luo, G.; Yang, W.; Li, Y.; Sun, J.; Zhan, K.; Liu, D.; Zhang, A. Genetic diversity, population structure and marker-trait associations for agronomic and grain traits in wild diploid wheat *Triticum urartu*. *BMC Plant. Biol.* **2017**, *17*, 112. [CrossRef]
10. Guzmán, C.; Alvarez, J.B. Molecular characterization of a novel waxy allele (Wx-A u 1a) from *Triticum urartu* Thum. ex Gandil. *Genet. Resour. Crop. Evol.* **2012**, *59*, 971–979. [CrossRef]
11. Zhang, Y.; Luo, G.; Liu, D.; Wang, D.; Yang, W.; Sun, J.; Zhang, A.; Zhan, K. Genome-, Transcriptome- and Proteome-Wide Analyses of the Gliadin Gene Families in *Triticum urartu*. *PLoS ONE* **2015**, *10*, e0131559. [CrossRef] [PubMed]
12. Talini, R.F.; Brandolini, A.; Miculan, M.; Brunazzi, A.; Vaccino, P.; Pè, M.E.; Dell'Acqua, M. Genome-wide association study of agronomic and quality traits in a world collection of the wild wheat relative *Triticum urartu*. *Plant J.* **2020**, *102*, 555–568. [CrossRef] [PubMed]
13. Miroshnichenko, D.; Ashin, D.; Pushin, A.; Dolgov, S. Genetic transformation of einkorn (*Triticum monococcum* L. ssp. *monococcum* L.), a diploid cultivated wheat species. *BMC Biotechnol.* **2018**, *18*, 68. [CrossRef] [PubMed]
14. Wang, K.; Shi, L.; Liang, X.; Zhao, P.; Wang, W.; Liu, J.; Chang, Y.; Hiei, Y.; Yanagihara, C.; Du, L.; et al. The gene *TaWOX5* overcomes genotype dependency in wheat genetic transformation. *Nat. Plants* **2022**, *8*, 110–117. [CrossRef]
15. Eudes, F.; Acharya, S.; Laroche, A.; Selinger, L.B.; Cheng, K.-J. A novel method to induce direct somatic embryogenesis, secondary embryogenesis and regeneration of fertile green cereal plants. *Plant. Cell Tissue Organ. Cult.* **2003**, *73*, 147–157. [CrossRef]
16. Chauhan, H.; Desai, S.A.; Khurana, P. Comparative analysis of the differential regeneration response of various genotypes of *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum*. *Plant. Cell Tissue Organ. Cult.* **2007**, *91*, 191–199. [CrossRef]
17. Alikina, O.; Chernobrovkina, M.; Dolgov, S.; Miroshnichenko, D. Tissue culture efficiency of wheat species with different genomic formulas. *Crop. Breed. Appl. Biotechnol.* **2016**, *16*, 307–314. [CrossRef]
18. Yang, S.; Xu, K.; Wang, Y.; Bu, B.; Huang, W.; Sun, F.; Liu, S.; Xi, Y. Analysis of biochemical and physiological changes in wheat tissue culture using different germplasms and explant types. *Acta Physiol Plant.* **2015**, *37*, 16128. [CrossRef]
19. Pellegrineschi, A.; Noguera, L.M.; Skovmand, B.; Brito, R.M.; Velazquez, L.; Salgado, M.M.; Hernandez, R.; Warburton, M.; Hoisington, D. Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. *Genome* **2002**, *45*, 421–430. [CrossRef]

20. Rasco-Gaunt, S.; Riley, A.; Cannell, M.; Barcelo, P.; Lazzeri, P.A. Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. *J. Exp. Bot.* **2001**, *52*, 865–874. [CrossRef]
21. He, G.Y.; Lazzeri, P.A. Improvement of somatic embryogenesis and plant regeneration from durum wheat (*Triticum turgidum* var. *durum* Desf.) scutellum and inflorescence cultures. *Euphytica* **2001**, *119*, 369–376. [CrossRef]
22. León, E.; Marín, S.; Barro, F. Improvement of in vitro culture response of elite wheat cultivars by selecting the source spike, the scutellum size and the temperature for the induction of embryogenesis. *Plant. Breeding* **2006**, *125*, 580–583. [CrossRef]
23. Tao, L.-L.; Yin, G.-X.; Du, L.-P.; Shi, Z.-Y.; She, M.-Y.; Xu, H.-J.; Ye, X.-G. Improvement of Plant Regeneration from Immature Embryos of Wheat Infected by *Agrobacterium tumefaciens*. *Agric. Sci.* **2011**, *10*, 317–326. [CrossRef]
24. Sabetta, W.; Crosatti, C.; Soltész, A.; Di Rienzo, V.; Montemurro, C. Screening Auxin Response, In Vitro Culture Aptitude and Susceptibility to *Agrobacterium*-Mediated Transformation of Italian Commercial Durum Wheat Varieties. *Molecules* **2016**, *21*, 1440. [CrossRef] [PubMed]
25. Miroshnichenko, D.; Chernobrovkina, M.; Dolgov, S. Somatic embryogenesis and plant regeneration from immature embryos of *Triticum timopheevii* Zhuk. and *Triticum kiharae* Dorof. et Migusch, wheat species with G genome. *Plant. Cell Tissue Organ. Cult.* **2016**, *125*, 495–508. [CrossRef]
26. Miroshnichenko, D.; Klementyeva, A.; Dolgov, S. The Effect of Daminozide, Dark/Light Schedule and Copper Sulphate in Tissue Culture of *Triticum timopheevii*. *Plants* **2021**, *10*, 2620. [CrossRef]
27. Wijerathna-Yapa, A.; Ramtekey, V.; Ranawaka, B.; Basnet, B.R. Applications of In Vitro Tissue Culture Technologies in Breeding and Genetic Improvement of Wheat. *Plants* **2022**, *11*, 2273. [CrossRef]
28. Miroshnichenko, D.; Klementyeva, A.; Pushin, A.; Dolgov, S. A competence of embryo-derived tissues of tetraploid cultivated wheat species *Triticum dicoccum* and *Triticum timopheevii* for efficient and stable transgenesis mediated by particle inflow gun. *BMC Plant. Biol.* **2020**, *20*, 442. [CrossRef]
29. Shrawat, A.K.; Armstrong, C.L. Development and Application of Genetic Engineering for Wheat Improvement. *Crit. Rev. Plant Sci.* **2018**, *37*, 335–421. [CrossRef]
30. Elhiti, M.; Stasolla, C. Transduction of Signals during Somatic Embryogenesis. *Plants* **2022**, *11*, 178. [CrossRef]
31. Bińka, A.; Orczyk, W.; Nadolska-Orczyk, A. The *Agrobacterium*-mediated transformation of common wheat (*Triticum aestivum* L.) and triticale (*x Triticosecale* Wittmack): Role of the binary vector system and selection cassettes. *J. Appl. Genet.* **2012**, *53*, 1–8. [CrossRef] [PubMed]
32. Hensel, G.; Marthe, C.; Kumlehn, J. *Agrobacterium*-Mediated Transformation of Wheat Using *Immature embryos*. *Methods Mol. Biol.* **2017**, *1679*, 129–139. [CrossRef] [PubMed]
33. Ishida, Y.; Tsunashima, M.; Hiei, Y.; Komari, T. Wheat (*Triticum aestivum* L.) transformation using immature embryos. *Methods Mol. Biol.* **2015**, *1223*, 189–198. [CrossRef] [PubMed]
34. Hayta, S.; Smedley, M.A.; Demir, S.U.; Blundell, R.; Hinchliffe, A.; Atkinson, N.; Harwood, W.A. An efficient and reproducible Agrobacterium-mediated transformation method for hexaploid wheat (*Triticum aestivum* L.). *Plant Methods* **2019**, *15*, 121. [CrossRef]
35. Abe, F.; Nakamura, S.; Mori, M.; Ashikawa, I. Low-temperature pretreatment of explants and high maltose concentration during callus culture improves particle-bombardment-mediated stable transgene expression in common wheat. *Plant. Biotechnol.* **2020**, *37*, 177–184. [CrossRef]
36. Qin, J.B.; Wang, Y.; Zhu, C.Q. Biolistic transformation of wheat using the HMW-GS *1Dx5* gene without selectable markers. *Genet. Mol. Res.* **2014**, *13*, 4361–4371. [CrossRef]
37. Ding, L.; Li, S.; Gao, J.; Wang, Y.; Yang, G.; He, G. Optimization of *Agrobacterium*-mediated transformation conditions in mature embryos of elite wheat. *Mol. Biol. Rep.* **2009**, *36*, 29–36. [CrossRef]