TDZ in Cereal Gametic Embryogenesis

7

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Abstract

Gametic embryogenesis is defined as the process that allows immature pollen grains – microspores – to parthenogenetically become embryos. The process can successfully be induced at a high frequency under in vitro culture conditions in a wide number of crop species. Microspores are haploid cells carrying half the somatic number of chromosomes, but if either spontaneously or artificially their chromosomal complement is doubled during the embryogenic pathway, the resulting embryos will become diploid and perfectly homozygous. The products of gametic embryogenesis are therefore called doubled-haploid plants, which are coveted materials for research and for plant breeding. Yet, to be efficiently used in a plant breeding program, doubled haploids need to be produced at a high frequency and in a reproducible manner. The efficiency and reproducibility of DH production are tied to the control of key factors intervening in the process. As is the case in many in vitro procedures, growth regulators play an important role in stimulating and guiding the process of orderly cell divisions leading to the regeneration of a complete plant from a single immature microspore. In this chapter, we review some of the key factors in this process with emphasis placed on the important role played by growth regulators, among which thidiazuron (TDZ). To illustrate the utility of TDZ in cereal gametic embryogenesis, we describe a highly efficient protocol for producing doubled haploids (either via anther culture or isolated microspore culture) that relies on an innovative combination of growth regulators: thidiazuron and dicamba. In our hands, this protocol proved successful for producing high numbers of barley, wheat, and rice doubled haploids.

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N. Ahmad, M. Faisal (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, https://doi.org/10.1007/978-981-10-8004-3_7

Keywords
Gametic embryogenesis · Cereals · In vitro culture · Doubled haploids · Thidiazuron · Dicamba

7.1 Overview

It was around 50 years ago that the first reports of doubled-haploid production in cereals started to be communicated in barley (Clapham 1973) and in rice (Guha-Mukherjee 1973). However, efficiency was then so low that these procedures could not make a meaningful contribution to breeding programs, and cereals were even considered recalcitrant to anther androgenesis (Foroughi-Wehr et al. 1976). Indeed, much higher success rates were being achieved in anther culture of species such as *Datura innoxia* and *Nicotiana tabacum*, where dozens of haploid plants could be easily obtained from just a few in vitro cultured buds (Guha and Maheshwari 1964; Nitsch and Nitsch 1969). In contrast, in barley, efficiencies of about 0.003–2 green plants per processed spike were being reported (Clapham 1973; Foroughi-Wehr et al. 1982; Foroughi-Wehr et al. 1984). In contrast, several years later very high rates of success were reported in several cereals such as wheat (Zheng et al. 2001) and barley (Kasha et al. 2001). Using isolated microspore culture in our lab, on a wide number of diverse barley F1 genotypes, we measured a mean efficiency of 111.5 green plants per processed spike (Esteves et al. 2014). This dramatic increase in efficiency of about 10,000% along these years can essentially be explained by two factors. One is the replacement of anther culture by isolated microspore culture, and the other is the successive improvements made to the protocol – including, among other parameters, changes to the type and concentration of growth regulators in culture media. Together, these lead to a very high frequency of microspore embryogenesis and green plant regeneration in cereals.

7.2 Introduction

7.2.1 Plant Hormones and In Vitro Culture of Plant Cells, Tissues, and Organs

Plant in vitro culture is a discipline which encompasses a very broad set of techniques and methods, all having in common the use of synthetic culture media onto which cells, organs, tissues, or pieces of plants, called explants, are cultured for specific purposes. The discipline started to develop after the Austrian plant physiologist Gottlieb Haberlandt (1902) hypothesized that plant cells are “totipotent,” meaning that they have the capacity to regenerate a complete individual under the proper circumstances and provided with adequate conditions in culture. Also, he stated that in order to regulate the proper division, growth, and differentiation of cultured cells, “growth factors” had to be provided. Amazing as it may seem, this

time, Haberlandt did not have the experimental means to test his hypothesis, but he was nonetheless beginning to establish the fundamentals of what was going to become the field of plant in vitro culture as a discipline (Murashige 1979). The reprogramming of young pollen grains (microspores) to produce embryos, also referred to as gametic embryogenesis or androgenesis, clearly confirmed Haberlandt's hypothesis about the totipotency of plant cells (Soriano et al. 2013). The process, in addition to its applied uses in breeding, provides an experimental system that allows a close insight into the causes and factors involved in the determination of fate and differentiation in plant morphogenesis. As mentioned above, from a practical point of view, DHs are exceptional resources either for research in plant genetics and genomics and to be exploited in crop breeding programs as well.

7.2.2 The Emergence of Doubled-Haploid Production

In higher plants, gametes are haploid cells where the normal, somatic number of chromosomes has been reduced by half. Embryos can be induced to form parthenogenetically from these haploid cells by a process called gametic embryogenesis (Olmedilla 2010). When male gametes are involved, the process is called "androgenesis," while "gynogenesis" describes the process where the female gametes are used (Germanà 2011). If a spontaneous doubling of the haploid number of chromosomes during the very early beginning of the embryogenesis process occurs, or if it is induced by treatment with chemical compounds such as colchicine, the resulting plants will carry perfectly identical copies of each of its chromosomes. It is this kind of product that is called a doubled haploid (DH). Because of their origin, DHs will be 100% homozygous, which means that genetic fixation can be obtained in the immediate progeny of an F1 plant. It is worth noting that between eight and ten cycles of selfing would be necessary to obtain a similar level of homozygosity (Dunwell 2010; Germanà 2011). A large population of DHs can be considered as a representative sample of the products of genetic recombination of their parental genes. Thus, DH production techniques allow the shortening of the length of a breeding cycle (from one cross to the next) by several years (Forster et al. 2007).

7.3 Obtaining Doubled Haploids

7.3.1 Current Methods for DH Production

Several methods for obtaining DHs have been developed in different species. For instance, interspecific crosses can lead to DH progeny following elimination of the set of chromosomes from the "exotic" parent (Kasha and Kao 1970), a method which is often used in barley and wheat (Devaux 2003). In maize, DHs are obtained by crosses made within the same species using as the male parent a genotype inducing haploidy (Prigge and Melchinger 2012). Also, pollinating with irradiated

pollen, as reported in watermelon by Sari and Abak (1994)), is another method used to obtain haploids. Alternatively, in vitro culture of ovaries – gynogenesis – has also been reported as a method for producing DHs, as in barley by Castillo and Cistué (1993), in *Beta vulgaris* by Bossoutrot and Hosemans (1985), in *Helianthus annuus* by Cai and Zhou (1984), and in *Allium cepa* by Campion and Alloni (1990). However, to the best of our knowledge, in none of these crops, the gynogenetic method for producing DHs reached the efficiency required to be of use in a breeding program. Compared to these alternatives, in vitro androgenesis – including both the methods of anther culture (AC) and of isolated microspore culture (IMC) – is often recognized as the most efficient and thus the preferred approach for producing DHs in crop species (Devaux and Kasha 2009).

7.3.2 Anther Culture and Isolated Microspore Culture: The Techniques

Anther culture was developed starting from the work of Guha and Maheshwari (1964, 1966) in obtaining haploid embryos of *Datura innoxia* Mill. when they cultured anthers in a defined culture medium. Clapham (1973) was the first to report the production of haploid plants of barley using AC. Next, by the late 1970s and throughout the 1980s, a huge amount of research was directed toward the improvement in the efficiency of androgenesis in cereals. From the very beginning of this period, the vast majority of this work focused on rice and on barley, and, a few years later, some varieties of these crops were considered as "model responsive genotypes" for both AC and microspore embryogenesis (Forster et al. 2007; Begheyn et al. 2016). Until today, there has been an impressive gain in efficiency in obtaining the androgenetic response (Devaux and Kasha 2009), and nowadays several efficient protocols are available (Jacquard et al. 2003; Cistué et al. 2003; Szarejko 2003).

Essentially, AC consists of first culturing the donor plants under optimal conditions of mineral nutrition, watering, lighting, temperature, and humidity. Next, at the proper stage of development of the microspores, stalks are harvested and spikes "pretreated" (Hoekstra et al. 1997) with a stress treatment, so that the microspores are stimulated to switch from the gametophytic pathway to the sporophytic pathway (Touraev et al. 2001; Devaux and Kasha 2009) (Fig. 7.1). Next, anthers are extracted from the florets and put in culture onto a defined induction medium, where embryos – often called "embryo-like structures" (ELSs) – might start to form inside the anthers containing responsive microspores. The ELSs are next recovered and transferred onto a regeneration medium where they will germinate and regenerate the DH plants (Fig. 7.2).

The IMC method to obtain DHs was developed more recently. Among the first reports, the work of Hunter (1988) in barley is one of the most exhaustive. As happened with AC, the efficiency of IMC was greatly improved in the following years. At present, this latter method is considered to significantly surpass AC and the *Bulbosum* method (interspecific cross) in its potential to produce DHs (Davies

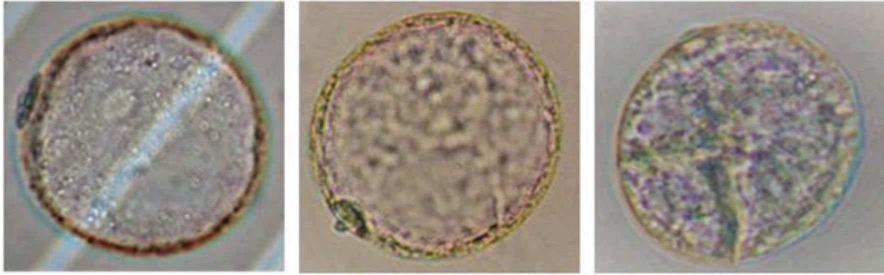

Fig. 7.1 Freshly isolated, non-pretreated microspore (left). After the pretreatment, embryogenic, competent microspores often display the "starlike" phenotype (center) (Maraschin et al. 2005) that characterizes them. After a few days in culture, divisions inside the embryogenic microspores become evident (right)

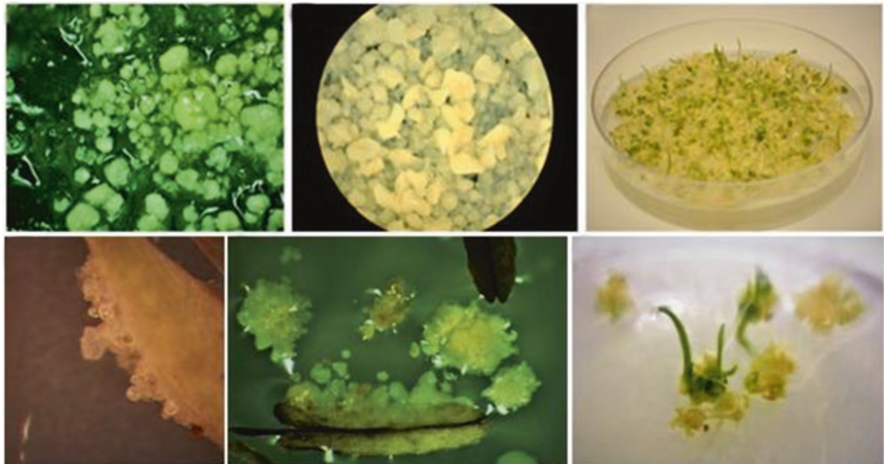

Fig. 7.2 A comparison of IMC (upper row: in rice to the left and barley at the center and to the right) and AC (row below: in wheat to the left and rice at the center and to the right). In IMC a massive production of ELSs is obtained, and both the control of the process and the manipulation of its products are much more facilitated. Both methods, in the three species mentioned here, were performed using induction medium containing TDZ and DIC

and Morton 1998; Li and Devaux 2005). In our own work (Esteves et al. 2014), we found IMC to be at least five times more efficient than AC in six-row spring barley. Indeed, IMC allows to plate large populations of selected, synchronized, viable, and embryogenic microspores (Li and Devaux 2005) through a series of steps of the protocol, including (1) blending the spikes, (2) filtering the produced extract through a 100 μm sieve, (3) rinsing several times the crude isolate of microspores, and (4) performing a discontinuous gradient centrifugation to greatly enrich for competent and viable microspores (Wenzel et al. 1975; Bedinger and Edgerton 1991) (Fig. 7.3). A detailed description of our IMC protocol is presented in Esteves et al. (2014).

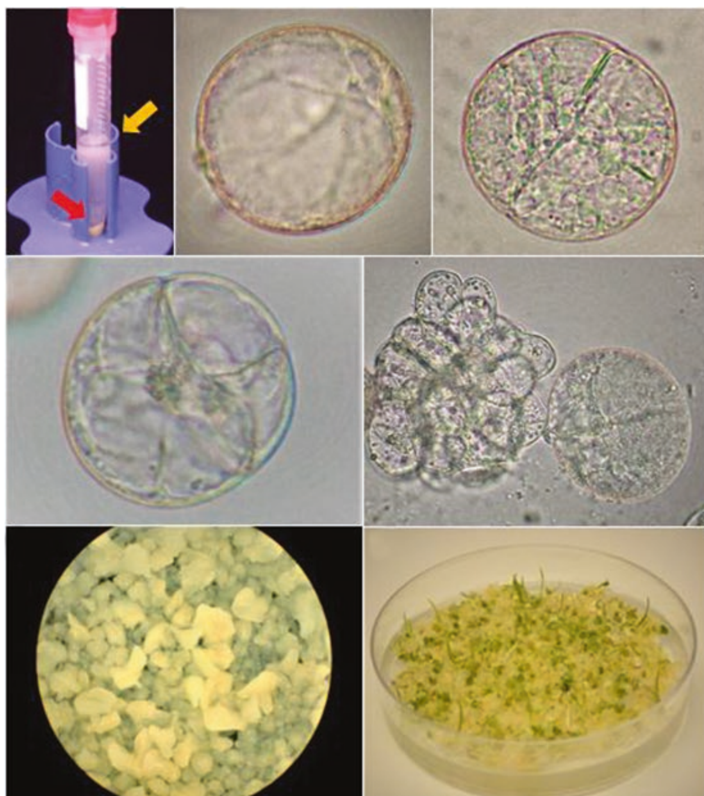

Fig. 7.3 Isolated microspore culture in barley performed with TDZ and DIC. Upper row, left: the product of the gradient centrifugation – a selection of viable, embryogenic microspores (orange upper arrow) and the debris (red arrow below). In the following panels (left to right), the development of microspores in culture is evident as they start dividing until, after 12–15 days, masses of ELSs are produced (bottom row, left). When the latter are transferred to a regeneration medium, these regenerate into green DH plants (bottom row, right)

Other protocols with slight differences are reported by Kasha et al. (2001) and by Maluszynski et al. (2003).

After harvesting the stalks and pretreating the spikes (microspores), only a small fraction of the microspores will engage in the sporophytic pathway. Among the differences existing between AC and IMC, one essential feature is that the latter, through the density gradient centrifugation, allows the selection of the fraction of viable microspores that are competent to engage in embryogenesis. At the same time, IMC allows a better follow-up and control of the cultures, because microspore embryogenesis is much better synchronized. For its part, AC requires less lab equipment and is more labor-intensive.

7.4 Gametic Embryogenesis: Impact of the Genotype

It is widely reported in the literature that success in obtaining the embryogenic response of microspores is strongly influenced by the genotype (Torp and Andersen 2009; Cistué et al. 1999; Marchand et al. 2008; Devaux and Kasha 2009; Makowska et al. 2015). Indeed, it has been proposed that microspore embryogenesis and plant regeneration might be under the control of separate classes of genes (Henry et al. 1994; Szakacs et al. 1988), as might be factors determining the regeneration of green or albino plants which very often are regenerated (Li and Devaux 2005; Weyen 2009). These are heritable traits (Foroughi-Wehr et al. 1984), highly influenced by the growth conditions of the donor plants and by the composition of the culture media (Kasha 1989; Knudsen et al. 1989; Simmonds 1989). Thus, for instance, Davies (2003) reported up to 1000 green plants regenerated per processed spike for a group of barley (winter-type) genotypes, while for spring-type barleys, the rate was only 10–20 green plants per spike. However, Atanassov et al. (1995) and Olmedilla (2010) identified factors of the protocol that, when optimized, lead to significant improvements in the production of DHs over genotypes displaying an otherwise poor response. The same has been observed in our lab when performing IMC on six-row spring barley, the type recognized as the most recalcitrant to in vitro androgenesis. We obtained a significant improvement in DH production when we optimized the pretreatment (30% increase in production) and the hormone regime used in the culture media (250% increase). Among the key changes in this regard were the use of thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea, TDZ) and dicamba (3,6-dichloro-O-anisic acid, DIC) as growth regulators in the induction medium. In both cases the gain was due both to a higher frequency of embryogenesis and a reduction in albinism as well. Also, by these means the response to androgenesis increased very significantly in almost all the genotypes processed, such that the proportion of F1s that yielded a useful number of DH lines exceeded 90% in our work. This is in stark contrast with the situation prior to these improvements, where only a subset (typically less than a third) of F1s responded sufficiently well in androgenesis to produce useful sets of progeny (Esteves et al. 2014). Moreover, we have recently obtained evidence suggesting that this very same protocol is also efficient in producing DHs of diverse genotypes of other species, such as wheat and rice (Esteves, unpublished).

7.5 In Vitro Controlling Factors in DH Production

7.5.1 Composition of the Basal Culture Medium

Optimizing culture media composition, both at the levels of the induction of microspore embryogenesis and of the regeneration phase, is an important aspect in androgenesis of cereals as it provides nutrition and also determines the fate of microspores (Santra et al. 2012; Mishra and Rao 2016). For instance, even though green plant regeneration is admittedly under strong genetic control, adjustments to

environmental conditions to which cells or explants in culture are exposed can change the genetic response (Zhou et al. 1991).

The composition of the basal culture medium for in vitro androgenesis typically consists of slight or more conspicuous modifications of the highly generalized MS medium (Murashige and Skoog 1962). Some of the commonly used basal media for the induction phase in AC or IMC in cereal species are the IMI medium for barley (Li and Devaux 2001); the N6 medium for rice, maize, and wheat (Chu et al. 1975); and the W14 medium for oats (Ouyang et al. 1989). Although the diverse species and genotypes usually show different basal medium requirements to induce plant regeneration from microspores, there are some major common improvements reported in the literature involving (1) the type of sugar (Cai et al. 1992; Navarro-Alvarez et al. 1994; Lentini et al. 1995), (2) the nitrogen source (Jähne and Lörz 1995; Raina and Zapata 1997; Lu et al. 2016), (3) the organic additives (Powell 1990; Hoekstra et al. 1992), and (4) the type and concentration of growth regulators (plant hormones) (Schulze 2007; Germanà 2011).

7.5.2 Growth Regulators and In Vitro Androgenesis

7.5.2.1 Hormones and In Vitro Gametic Embryogenesis

Plant growth regulators and hormones are known as key signaling molecules controlling plant growth and development, as well as initiating signal transduction pathways in response to environmental stimuli (Kohli et al. 2013). The plant hormones, mainly auxins and cytokinins, are known to control the dedifferentiation process in plant in vitro cultures, and the rate of success can be enhanced by manipulating the plant growth regulators (Mishra and Rao 2016). Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-acetic acid (NAA) are the most commonly used growth regulators for the induction of callus from rice anthers (Trejo-Tapia et al. 2002). However, neither 2,4-D nor NAA can support regeneration, and the use of cytokinins like kinetin (KIN) and 6-benzyl-aminopurine (BAP) is required (Mandal and Gupta 1995). In barley AC, weaker auxins at low concentration have been commonly used, but in some cases the auxin has been omitted, leaving the cytokinin BAP as the sole growth substance (Hunter 1988; Kihara et al. 1994). In addition, Huang (1984) found that another type of hormone, abscisic acid (ABA), may promote barley green plant regeneration. Hoekstra et al. (1997) stated that the right hormone in the culture medium of microspores is necessary for the induction of plant production, and in barley they achieved this either with the application of a cytokinin (BAP) or 2,4-D in the culture medium. Alternatively, Kasha et al. (2001) using IMC in barley, and Ziauddin et al. (1992) using AC and IMC in barley and wheat, included the naturally occurring auxin phenylacetic acid (PAA) together with BAP in the induction medium and the cytokinin only in the regeneration medium, and they both improved significantly the production of ELSs and green DH plants. In our lab we tried to reproduce this PAA-based protocol, without success. It has been proposed that the action of PAA is dependent on plant growth

and its associated endogenous levels of auxin (Kasha et al. 2001), and thus more experimentation might be necessary to optimize it use.

While there are some reports suggesting that growth regulators are not essential for microspore embryogenesis in most plant species (Olmedilla 2010), it has also been reported that recalcitrant and low-responding wheat and barley genotypes became responsive following a pretreatment with chemicals, including growth regulators (Liu et al. 2002), or by changing the hormone composition of the culture medium (Esteves et al. 2014). Aligned with these results, Shariatpanahi et al. (2006) obtained a significantly higher frequency of embryogenesis and higher green plant regeneration from wheat microspores when growth regulators were included in the induction medium, compared to when they were not. Either alone or in combination, various types of auxins and cytokinins have been used in media designed for in vitro androgenesis (Cistué et al. 1999; Devaux and Pickering 2005; Datta 2005), and the inclusion of these plant growth regulators has been shown to modify embryogenesis and plant regeneration frequencies (Tyankova and Zagorska 2008).

At the beginning of the development of IMC in barley, Hunter (1988) used an induction medium containing only 1.0 mg/l of the auxin IAA (indoleacetic acid) as growth regulator, and he compared it to five others containing 1.0 mg/l of the cytokinins [BAP, KIN, zeatin (ZEA), zeatin riboside (ZEAr), or 6-γ-γ-(dimethylallylamino)-purine (2iP)]. He concluded that there existed differences in androgenesis that were attributable to this factor, and it was BAP that provided the best result. Then, to a medium containing 1.0 mg/l of BAP, he compared four other media containing one of our auxins, either IAA, indole butyric acid (IBA), NAA, or 2,4-D, and concluded that IAA provided the best response. Yet, as he subsequently found a negative correlation between the concentration of IAA (between 0 and 1.0 mg/l in the medium) and the regeneration of green plants, he concluded that an optimal induction medium would only contain the cytokinin BAP at a rate of 1.0 mg/l. He named this developed induction medium as FHG, which was to become one of the most commonly used in androgenesis in barley all over the world (Devaux and Pickering 2005).

As described above, the effects of plant growth regulators in in vitro androgenesis have been considered controversial (Devaux and Pickering 2005). However, based on our own experience, hormone composition of induction and regeneration media in in vitro androgenesis is a key success factor in obtaining high frequencies of embryogenesis and the regeneration of green DH plants. Indeed, when we included TDZ as a growth regulator in the induction medium, together with the auxin DIC, a massive embryogenic response was obtained, and at the same time, both the total number of green regenerated plants and the ratio of green to albino plants increased dramatically compared to the standard BAP-based induction medium (Esteves et al. 2014).

7.5.2.2 The Role of Cytokinins in Gametic Embryogenesis

If the impact of growth regulators in inducing gametic embryogenesis has been a matter of discussion in the literature, to a certain degree, it is much less so in the case of somatic embryogenesis. Somatic embryogenesis is the process by which

diploid, somatic plant cells differentiate into somatic embryos. Any differentiated plant cell that retains its nucleus has the ability to revert to the embryogenic condition and regenerate an entire plant (Reynolds 1997). Somatic embryo formation usually requires first a treatment of diploid cells with plant hormones, mostly auxin but also cytokinins, under specific culture conditions, and later auxin withdrawal to allow embryogenesis to continue (Toonen et al. 1994; Cistué and Kasha 2005). Indeed, in the majority of species studied where exogenous plant growth regulators were necessary for inducting somatic embryogenesis and embryoid formation, both auxins and cytokinins were shown to be key factors in determining the response (Jimenez 2005).

Plant regeneration, regardless of ploidy level, occurs by either somatic embryogenesis or organogenesis (Rybczynski et al. 1991). It has been proposed that both types of embryogenesis, somatic and gametic embryogenesis, are regulated by the same basic cellular mechanisms, namely, cell expansion and asymmetric cell division (De Jong et al. 1993). Also, signal molecules, such as hormones and growth regulators, have been shown to play a role during both types of embryogenesis (Dodeman et al. 1997). Considering that both TDZ and DIC are reported to be efficient growth regulators capable of inducing direct and indirect somatic embryogenesis, we decided to experiment with them in gametic embryogenesis in barley via IMC. The experiments were to be carried out in two steps: The first was to evaluate replacing the classically used cytokinin BAP (at 1.0 mg/l) by TDZ at a range of concentrations (0.1, 0.3, and 1.0 mg/l). The second step focused on the impact of replacing BAP by both TDZ (included at the concentration which had performed best in the first step) and the auxin DIC at a range of concentrations (0.1, 0.3, and 1.0 mg/l). Four contrasting genotypes were chosen for this experiment: one winter, two-row type (Igri); one spring, two-row type (Gobernadora); and two spring, six-row types (ACCA and Léger). Our results showed that when BAP was replaced by TDZ alone, we did not observe statistically significant differences for the parameters tested (total number of green plants, total number of albino plants, and green/albino plants ratio; Table 7.1). BAP and TDZ have very similar molecular weights (225.25 and 220.25 g/mol, respectively), so this taught us that even at 1/10 of its molar concentration, TDZ was as potent as BAP in inducing embryogenesis and ELS formation. Next, contrasting results were observed during the second step of the experiment: when BAP was replaced by the combination of TDZ (0.3 mg/l) and DIC (1.0 mg/l), the number of regenerated green plants increased consistently, and significantly, with increasing concentrations of DIC (Table 7.1). Averaged over the four barley cultivars, all three induction media including DIC and TDZ produced significantly more green plants than the BAP check. Indeed, relative to the control, the TDZ (0.3 mg/l) + DIC (1.0 mg/l) treatment produced greater than five times more green plants on average (176.0 vs. 34.4 green regenerated plants/10^5 microspores, respectively; $P < 0.01$), whereas the number of albino plants produced did not change significantly (197.1 vs. 162.4; $P = 0.249$). Thus, the observed increase in the total number of plants/10^5 microspores (373.1 vs. 196.8, $P < 0.001$) could be almost entirely ascribed to an increase in the production of green plants. As can also be seen in Table 7.1, the green/albino plant ratio increased dramatically

Table 7.1 Comparison of the impact of replacing BAP with three levels of TDZ (first phase of the experiment) and by 0.3 mg/l of TDZ + DIC (three levels of DIC; second phase) in induction medium of barley IMC

Growth regulator (mg/l)	Green plants	Albino plants	Total plants	Green/albino plants
First phase				
BAP 1.0	33.2[a]	161.2[a]	194.4[a]	0.21[a]
TDZ 0.1	42.1[a]	143.1[a]	185.2[a]	0.29[a]
TDZ 0.3	40.1[a]	133.4[a]	173.5[a]	0.30[a]
TDZ 1.0	37.5[a]	101.5[a]	138.5[a]	0.37[a]
Second phase				
BAP 1.0	34.4[a]	162.4[a]	196.8[a]	0.21[a]
TDZ 0.3 + DIC 0.1	66.9[b]	173.6[a]	240.5[a]	0.38[ab]
TDZ 0.3 + DIC 0.3	107.0[c]	178.8[a]	285.8[b]	0.60[bc]
TDZ 0.3 + DIC 1.0	176.0[d]	197.1[a]	373.1[b]	0.89[c]

Numbers in the table correspond to regenerated plants per 10^5 microspores plated. Different letters correspond to significant ($P < 0.05$) differences

from 0.21 for the control treatment up to 0.89 for the TDZ 0.3 mg/l+DIC 1.0 mg/l treatment ($P < 0.01$). Since the mean number of albino plants was not significantly different among all the treatments, it is clearly the increasing number of green regenerated plants, produced at increasing concentrations of the auxin, that allowed the green/albino plant ratio to increase significantly (5.1-fold).

7.5.2.3 TDZ and In Vitro Embryogenesis

In cereal somatic embryogenesis, TDZ, in combination with the auxins 2,4-D and NAA, has been shown to improve more than threefold the response of recalcitrant indica rice varieties when compared to some other cytokinins such as ZEA, BAP, KIN, and 2iP (Wenzhong et al. 1994). Improved regeneration frequencies were also described for Australian rice varieties when combining TDZ with the auxin NAA, instead of using BAP alone, for mature embryo-derived callus production (Ahzria and Bhalla 2000). Similarly, using mature embryos as explants, Parmar et al. (2012) reported that a considerable improvement in the regeneration frequency (up to 97%) and in the average number of shoots was obtained with a combination of TDZ and 2,4-D.

Although TDZ has proved useful in inducing somatic embryogenesis and even allowed improving the response of recalcitrant species in several instances, it has seldom been tested in gametic embryogenesis in cereal species, and the only existing reports in the literature proved either unsuccessful or nonconclusive. For instance, Ouédraogo et al. (1998) reported that the addition of 0.01 mg/l of TDZ in AC of *Hordeum vulgare* increased or decreased the number of embryos per 100 anthers, or it had no effect, depending on the cultivar. Also, Kiviharju et al. (2005) added TDZ as a supplement in induction media in AC of oats (0.2 and 0.5 mg/l) and found no benefits or even a reduction of the regeneration efficiency in relation to the other cytokinins such as ZEA, 2iP, KIN, and BAP. In fact, at the same concentrations as TDZ, the latter cytokinins performed much better in embryogenesis and green plant

regeneration. In contrast with these previous results, in our lab, after a step-by-step adjustment of the optimal concentrations, we found that 0.3 mg/l of TDZ together with 1.0 mg/l of DIC in the induction medium dramatically increased the yield of green plants/spike, by simultaneously increasing the frequency of both gametic embryogenesis and green plant regeneration and reducing albinism as well. Further experimentation leads us to reduce the DIC concentration in the induction medium to 0.6 mg/l as, at the higher dose, regenerated plants seemed to suffer from vitrification. Using this latter formulation (TDZ at 0.3 and DIC at 0.6 mg/l) allowed us to produce hundreds of DHs of a wide array of recalcitrant barley genotypes in a reproducible fashion over several years, and this same induction medium has recently proved efficient to obtain massive embryogenesis and plant regeneration in wheat and rice (unpublished results).

7.6 Conclusion and Perspectives

It has been recently proposed that plant growth regulators, which cross talk and interact with the plant's genotype and environmental factors, play a crucial role in microspore embryogenesis, controlling microspore-derived embryo differentiation and development as well as haploid/doubled-haploid plant regeneration (Żur et al. 2015). In agreement with this, we observed a significant positive impact in gametic embryogenesis of cereal species provided by modifying the types and concentrations of auxins and cytokinins in the induction medium. To the best of our knowledge, this is the first report of a substantial benefit of the inclusion of TDZ, together with the auxin DIC, in a gametic embryogenesis protocol. Replacing the traditionally used cytokinin BAP used alone by the cytokinin TDZ + the auxin DIC allowed not only to nearly double the total number of regenerated plants but also increased by 4.1-fold the ratio of green/albino plants. Even if it is well known that cytokinins are involved in the control of chloroplast biogenesis and function (Zubo et al. 2008) and also in chlorophyll synthesis (Ricci et al. 2001), the role played by the auxin-cytokinin ratio in increasing the proportion of green regenerated plants is not clear and would deserve further research (i.e., it remains to be determined whether the same kind of improvement would be seen using other types of auxin-cytokinin combinations). Nonetheless, we observed that this same hormone formulation, including TDZ and DIC, proved useful to obtain high numbers of green DHs in a diverse array of barley genotypes and in rice and wheat as well, using either AC or IMC. Thus, we believe that the adjustment of the ratio and dosage of this highly efficient combination of auxin and cytokinin (TDZ and DIC) might be a key to overcoming the recalcitrance to DH production in other species and genotypes, such as indica races of rice and leguminous, woody, and fruit tree species. More research would be warranted to test this hypothesis, which would speed up breeding projects dealing with these species. Also, such research might shed further light on the sporophytic pathway leading to DH plants.

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