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Improvements in Cereal Tissue Culture by Thidiazuron: A Review

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ABSTRACT

This article reviews research to establish highly regenerative cell and tissue culture systems in cereals and grasses as a prerequisite for crop improvement using biotechnological methods. The strategies were described in the historical framework of investigations in this field and then new approaches were discussed focussing on the considerable recent progress made using the cotton defoliant thidiazuron (TDZ) for enhancement of morphogenic competence in the *Poaceae* during the last 15 years. In 1982, TDZ was first described to have cytokinin-like activity and subsequently, numerous studies have characterized this compound as a powerful plant growth regulator for dicots with special emphasis on recalcitrant leguminous and woody species. However, TDZ was evaluated for cereal tissue culture with a remarkable delay. The results summarized clearly showed that TDZ can significantly improve morphogenic response from callus derived from a wide range of explants concerning frequency of shoot formation, number of shoots per explant and the time needed for shoot induction compared to other cytokinins. Moreover, TDZ has been used effectively for establishment of shoot meristematic cultures from model and agronomically important cultivars using shoot apices, shoot meristematic segments as well as nodes and pronounced differences were obtained regarding shortening of the time frame especially for barley and wheat. Besides that, high frequency plant regeneration with long-term retention of morphogenecity in a relatively genotype-independent manner was observed. Nevertheless, in a few cases problems associated with the use of TDZ were noted. The data summarized imply that TDZ is also a potent growth regulator for cereals which (i) further minimizes the recalcitrant nature of the *Poaceae* and which (ii) extends the application of transformation protocols to elite genotypes and to more readily available explants.

Keywords: genotype dependency, *in vitro* morphogenesis, long-term regeneration, plant growth regulators, *Poaceae*, shoot meristematic cultures, transformation

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **2iP**, 6-γ-γ-(dimethylallylamino)-purine; **BAP**, benzylaminopurine; **IAA**, indole-3-acetic acid; **Kin**, kinetin; **NAA**, α-naphthaleneacetic acid; **TDZ**, thidiazuron; **Zea**, zeatin

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INTRODUCTION

The first experiments to culture plant cells under *in vitro* conditions were conducted more than one hundred years ago (Haberlandt 1902). Those experiments had no success since, among others, there was no knowledge about plant growth substances. It took decades until their detection, isolation and subsequently the observation made by Skoog and Miller (1957) on the auxin/cytokinin ratio controlling root and/or shoot formation from tobacco (*Nicotiana tabacum*) pith tissue cultures *in vitro*, being a milestone for the development of plant tissue and cell culture. However, more than twenty years after that breakthrough and promising results with dicots, success with monocots, especially with the cereals was rare. The situation was summarized in a lecture delivered to the first Annual Meeting of the French Section, International Association for Plant Tissue Culture by King *et al*. (1978). From their experience with *Zea mays* and *Sorghum bicolor*, and from an extensive examination of the literature, the authors concluded that none of the technology essential for *in vitro* genetics had yet been satisfactory established with cereal species. Nonetheless, efforts did not

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Invited Review

fail due to the high economic features of the cereals.

Genetic improvement of the major cereals such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*) and barley (*Hordeum vulgare*) but also sorghum (*Sorghum bicolor*), millet (*Pennisetum* sp.), oat (*Avena sativa*) and rye (*Secale cereale*) has been particularly important for plant breeders for decades, since these crops provide more than half of the food consumed by mankind being the main sources of plant proteins and carbohydrates (FAO 2007). In addition, these species are the basis for production of animal feed, oils, starch, flour, sugar, processed foods, malt, alcoholic beverages, gluten and renewable energy (Heiermann *et al.* 2002). The increase in productivity as a result of the "Green Revolution" in combination with intensified crop management, greater inputs of fertilizer and irrigation met the demands until the 1980s (Hedden 2003). Since then, growth rates of yields have slowed down due to declining resources of water and arable land, deteriorating soil conditions as a result of general environmental degradation (Huang *et al.* 2002) as well as due to limitations in the germplasm pool (McIntosh 1998; Jauhar 2003). To ensure the world's food supply for the future with a continued population growth up to 8 billion people in 2025 will demand for cereals to increase by 41% between 1993 and 2020 to reach 2.490 million metric tons (Dyson 1999; Pinstrup-Anderson 2001).

To meet that dramatic increase in cereal demand worldwide, new approaches and technologies also for generating new varieties are necessary. The rapidly developed methods of molecular and genetic engineering provide powerful and novel means to supplement and complement the traditional methods of plant improvement (Kishore and Shewmaker 1999; Sairam and Prakash 2005). Nowadays, nearly thirty years after the above mentioned frustrating statement of King *et al.* (1978), not only were transgenic cereals generated (Shewry and Jones 2005; Bajaj and Mohanty 2005; Shrawat and Lörz 2006; Dahleen and Manoharan 2007; Vasil 2007), several of which are in field tests (e.g. Horvath *et al.* 2001; Barro *et al.* 2003; Shewry *et al.* 2006) while genetically engineered maize was grown on 108.5 millions of hectares in 13 countries in 2006 (Clive 2006). This enormous success is based on rapidly developed methods in plant biotechnology, a discipline requiring both cell and molecular biology. While techniques for gene isolation, DNA delivery, availability of reporter and selectable genes, strategies to enhance gene expression e.g. by the use of introns, analysis of integration and expression displayed a dramatic development in the last 25 years (Birch 1997; Kohli *et al.* 2003), in comparison advances in plant and cell culture of the cereals were moderate due to limitations like genotype, explant type and short regeneration ability. In spite of the molecular advances, transformation efficiency is low due to the bottleneck represented by the tissue culture performance (Janakiraman *et al.* 2002; Shrawat *et al.* 2003) which is reflected by a vast amount of literature concerning optimisation of regeneration protocols also in very narrow time frame.

This review focuses on new developments in cereal tissue culture with special emphasis on barley and wheat allowing now high efficient regeneration of plants in a genotype- and explant-independent manner through the use of the potent cytokinin-like substance thidiazuron (TDZ). Exactly 50 years after the breakthrough made by Skoog and Miller (1957) much was achieved and the often mentioned recalcitrance of cereals and grasses in tissue culture was further reduced.

STRATEGIES TO ESTABLISH REGENERATIVE CULTURE SYSTEMS IN CEREALS

Search for suitable donor explants

In the first investigations to establish tissue culture systems from cereals, mature seeds, isolated mature embryos and tissues derived from young seedlings were evaluated. Whereas in dicot species leaves from *in vitro* as well as *ex vitro* grown plants are the most common source for callus initiation and subsequent plant regeneration, the use of these easily available explants was found not to be successful for cereals in early reports. Callus formation from segments of young leaves and leaf base, respectively, was described but regeneration of plants failed or was very rare (O'Hara and Street 1978; Saalbach and Koblitz 1978). However, growth of leaves occurred at the basal part requiring high mitotic activity typical for meristematic regions. The expected ability of young leaf tissues of graminaceous species to express morphogenic capacity could be demonstrated first for sorghum (Wernicke and Brettell 1980; Wernicke *et al.* 1982), napier grass (*Pennisetum purpureum*) (Haydu and Vasil 1981), guinea grass (*Panicum maximum*) (Lu and Vasil 1981), orchard grass (*Dactylis glomerata*) (Hanning and Conger 1982) but also for rice (Wernicke *et al.* 1981) and wheat (Ahuja *et al.* 1982; Zamora and Scott 1983). However, a strong basipetal gradient in wheat leaves was observed concerning response to 2,4-dichlorophenoxyacetic acid (2,4-D) and plant regeneration was only achieved in a few genotypes out of 21 tested and only from very immature tissue (Wernicke and Milkovits 1984).

Another easily available tissue analysed was the shoot apical meristem which contains the dome of actively dividing cells thus being promising for establishing morphogenic cultures. The suitability of these explants was already demonstrated in 1975 by Cheng and Smith describing shoot formation up to 85% from callus derived from apical meristems isolated from one week-old barley seedlings. However, further assessment of these explants revealed contradictory results. Whereas Koblitz and Saalbach (1976) as well as Dale and Deambrogio (1979) described rare or no shoot formation, the initiation of tissue cultures capable of plant regeneration via somatic embryogenesis in barley (Weigel and Hughes 1985) and organogenesis in wheat was reported (Wernicke and Milkovits 1986).

In addition, roots from young wheat and barley seedlings were tested for morphogenic ability, but the easily induced callus was only capable of root formation (Bhojwani and Hayward 1977; Chin and Scott 1977a; Chawla 1989). Moreover, mesocotyls were also evaluated for culture initiation and plant regeneration, but efficiency was low (Jelaska *et al.* 1984; Bartók and Sági 1990).

Mature seeds or embryos from all cereals were intensively analysed and originated tissue cultures as could be shown for rice (Nishi *et al*. 1968; Nakano and Maeda 1979), maize (Harms *et al.* 1976), oats (Carter *et al.* 1967; Cure and Mott 1978), wheat (Cure and Mott 1978) and barley (Bayliss and Dunn 1979; Lupotto 1984). Nevertheless, mature embryos often gave soft watery callus with low regeneration efficiency declining after the first subcultures when directly compared to immature embryos (Rotem-Abarbanell and Breiman 1989; Rikiishi and Yasuda 1994; Ganeshan *et al.* 2003; Zale *et al.* 2004).

Immature inflorescences, which are smaller than 2 cm and completely covered by the leaves, were also evaluated for their morphogenic response. First successful plant regeneration was reported for sorghum (Brettell *et al.* 1980), Italian ryegrass (*Lolium multiflorum*) (Dale *et al.* 1981) and napier grass (Wang and Vasil 1982). The suitability of these explant sources was confirmed for wheat (Ozias-Akins and Vasil 1982; Maddock *et al.* 1983; Rajyalakshmi *et al.* 1988; Sharma *et al.* 1995) and barley (Thomas and Scott 1985), too. However, despite immature inflorescences being well recognized as a source of totipotent cultures, only a limited number of reports are available.

Anthers were predominantly cultured to generate haploid plants, which can develop via direct embryogenesis from the microspores or via plant regeneration from the callus derived from these explants. Despite first successful reports in rice (Iyer and Raina 1972; Chen 1978), wheat (Ouyang *et al.* 1973) and barley (Clapham 1973; Foroughi-Wehr *et al*. 1982), it took years of optimisation of the techniques to achieve efficient green plant regeneration in all

Table 1 Plant regeneration from tissue cultures of barley with special focus to the explant source.

Explant source[1]	Barley
apical meristem/ shoot apex	Cheng and Smith 1975; Wilson *et al*. 1976; Koblitz and Saalbach 1976; Weigel and Hughes 1985; Rengel and Jelaska 1986; Vitanova *et al*. 1995; Zhang *et* al. 1998; Bregitzer *et al*. 2002; Ganeshan *et al*. 2003; Sharma *et al*. 2004; Zapata *et al*. 2004; Ganeshan *et al*. 2006a
leaf base	Saalbach and Koblitz 1978; Mohanty and Ghosh 1988; Becher *et al*. 1992; Ruíz *et al*. 1992; Pasternak *et al*. 1999; Ganeshan *et al*. 2003
immature embryos	Norstog 1970; Dale and Deambrogio 1979; Deambrogio and Dale 1980; Powell *et al*. 1983; Kott and Kasha 1984; Hang and Franckowiak 1984; Seguin-Swartz *et al*. 1984; Thomas and Scott 1985; Hanzel *et al*. 1985; Breiman 1985; Goldstein and Kronstad 1986; Ahloowalia 1987; Chawla and Wenzel 1987; Lührs and Lörz 1987; Mathias and Mukasa 1987; Kott *et al*. 1987; Gaponenko *et al*. 1988; Komatsuda *et al*. 1989; Rotem-Abarbanell and Breiman 1989; Pickering 1989; Ziauddin and Kasha 1990; Ryschka *et al*. 1991; Bregitzer 1992; Ruíz *et al*. 1992; Golds *et al*. 1993; Huang *et al*. 1993; Baillie *et al.* 1993; Wang *et al.* 1993; King and Kasha 1994; Kachhwaha and Kothari 1994; Rikiishi and Yasuda 1994; Bregitzer *et al.* 1995; Oka *et al*. 1995; Walmsley *et al*. 1995; Dahleen 1995; Kachhwaha and Kothari 1996; Kachhwaha *et al*. 1997; Singh *et al*. 1997; Castillo *et al*. 1998; Jiang *et al.* 1998; Barro *et al.* 1999; Dahleen 1999; Dahleen and Bregitzer 1999; Shan *et al.* 2000; Nuutila *et al.* 2000; Dahleen and Bregitzer 2002; Ganeshan *et al*. 2003; Chang *et al*. 2003; Przetakiewicz *et al.* 2003; Rikiishi *et al*. 2003; Eudes *et al*. 2003; Chauhan and Kothari 2004; Halámková *et al.* 2004; Tiidema and Truve 2004; Biagioli *et al*. 2006; Ganeshan *et al*. 2006b; Jha *et al.* 2007
mature seeds/mature embryos	Lupotto 1984; Rengel 1987; Ukai and Nishimura 1987; Kothari and Chandra 1988; Rotem-Abarbanell and Breiman 1989; Huang *et al*. 1993; Kachhwaha and Kothari 1994; Rikiishi and Yasuda 1994; Oka *et al*. 1995; Akula *et al*. 1999; Ganeshan *et al*. 2003; Zapata *et al*. 2004; Sharma *et al.* 2005b; Ganeshan *et al*. 2006a; Park *et al.* 2006
mesocotyl	Jelaska *et al*. 1984; Müller and Wegner 1989
immature inflorescences	Thomas and Scott 1985; Barro *et al.* 1999; Havrlentová *et al*. 2001
roots	Chand and Sahrawat 2000
coleoptile	Sahrawat and Chand 2004
nodes	Sharma *et al*. 2007

[1]Anthers and microspores were not included since majority of the studies was focussed on obtaining doubled haploids in breeding programs for practical application.

cereal species (reviewed in Devaux and Pickering 2005). Based on the success obtained with anthers, isolated microspores were also cultured. Due to extensive work and several important modifications reproducible regeneration systems applicable for production of homozygous plants were established especially in barley (Jähne and Lörz 1995; Lantos *et al.* 2005) which can also be used for gene transfer.

Furthermore, immature embryos isolated from young ears 7 to 15 days after anthesis, when the caryopsis is still green and in a milky stage, were evaluated as an explant source. Their morphogenic competence was first described in maize by Green and Philipps (1975). Comparing different kinds of explants, it was demonstrated that plant regeneration can be obtained easily from immature embryos (e.g. Chin and Scott 1977b; Dale and Deambrogio 1979; Gosch-Wackerle *et al.* 1979; Rybczynski 1980). Since then, efforts to establish embryogenic cultures concentrated on the use of immature embryos in all cereals and grasses. Numerous successful reports repeatedly confirm the suitability of these explants as it is documented for barley as an example (**Table 1**). Histological analysis demonstrated that the callus capable of embryoid and plant formation originates from the scutellum involving the scutellum's three basic tissue systems: dermal, ground and vascular (Lu and Vasil 1985; Magnusson and Bornman 1985; Ryschka *et al.* 1991). This embryogenic callus can be visually identified due to its nodular dense structure and yellow-white colour (Ozias-Akins and Vasil 1982).

In the second half of the eighties the identification of tissue types, which are competent for regenerating whole plants run into the general consent that immature tissues and cells derived from a source close to the embryonic state, are the most suitable explants. Subsequently, for all the important cereals, efficient and reproducible plant regeneration systems have been developed based on immature embryos (Vasil 1987; Lörz *et al.* 1988). Nevertheless, one of the main problems linked to the immature embryos but also to the immature inflorescences as well as anthers/microspores is the need for continuous growth of donor plants. These plants have to be grown under controlled environmental conditions without pathogens and pesticides requiring intensive labour, time and space. In addition, a seasonal influence of harvesting time on the tissue culture response of immature embryos (Dahleen 1999; Sharma *et al.* 2005a) and on haploid production (Foroughi-Wehr and Mix 1979; Ritala *et al.* 2001) has been shown. Thus, efforts did not fail to re-evaluate the morphogenic potential of alternative explants despite earlier studies revealing that efficiency of regeneration is low and of low reproducibility. Another impulse came from the development of the particle gun allowing now the introduction of foreign genes in tissues competent for regeneration (Klein *et al.* 1987). Subsequently, a number of reports considerably increased in the last two decades successfully exploring alternative tissues, as summarized in **Table 1** for barley as an example.

Factors associated with regenerative competence

Independent of the explant source used, the establishment of morphogenic cultures is considerably influenced by several factors. Efficiency of the regeneration protocols is limited by a strong genotype dependency as could be observed for leaves (Wernicke and Milkovits 1984; Hanning and Conger 1986; Ruíz *et al.* 1992), mature embryos (Bayliss and Dunn 1979; Lazar *et al.* 1983; Zale *et al.* 2004; Bi *et al.* 2007) and immature inflorescences (Maddock *et al.* 1983; Rajyalakshmi *et al.* 1988). A strong genotype specifity was also obtained for anthers and microspores (Powell 1988; Kuhlmann and Foroughi-Wehr 1989; Logue *et al.* 1993).

Since considerable attention has been given to immature embryos there are numerous reports describing genotypic effects and here only a few examples are mentioned for barley and wheat (e.g. Maddock *et al.* 1983; Hanzel *et al.* 1985; Goldstein and Kronstad 1986; Ahloowalia 1987; Lührs and Lörz 1987; Redway *et al.* 1990; Hess and Carman 1998; Viertel *et al.* 1998; Varshney and Altpeter 2001). Remarkable data have been accumulated revealing that tissue culture ability is under genetic control. However, knowledge about the genetic control mechanisms on *in vitro* response is still insufficient (Bregitzer and Campbell 2001; Tyankova and Zagorska 2001). Thus routine application of gene transfer to improve traits in a desired cultivar is strongly limited, since a high efficient and reproducible regeneration system is only available for a few so-called model genotypes. Whereas for generation of transgenic rice plants genotype dependency seems not to be so strong since 40 genotypes have been transformed (Cheng *et al.* 2004), success in transformation of other cereals is closely related

to the model genotypes, for example 'Bobwhite' and 'Fielder' for wheat or 'A188' and its hybrids for maize. The crucial importance of the genotype is convincingly demonstrated for barley. From nearly seventy studies on generation of transgenic plants being available in two thirds of all reports the model cultivars 'Golden Promise' for immature embryos and 'Igri' for microspores were used which worked for microprojectile- as well as for *Agrobacterium*-mediated transformation (**Table 2**).

In the case of explants originating from *ex vitro*-grown plants, the *in vitro* response is considerably affected by the growth conditions of the donor plants. Abiotic and biotic stresses reduce embryogenic competence of immature embryos as well as microspores since such stresses may influence the endogenous hormone level and balance. Donor-plant environment effects were repeatedly reported (Carman *et al.* 1987; Lührs and Lörz 1987; Dahleen 1999) and a promoting influence of cooler growth conditions on tissue culture response was shown for microspores (Lyne *et al.* 1986; Afele *et al.* 1992) and immature embryos (Carman *et al.* 1987; Pickering 1989). A correlation between endogenous hormone content and embryogenic competence was first described by Carnes and Wright (1988). Measurements of phytohormones in kernels of three varieties of maize differing in their embryogenic reaction revealed a 16- and 20-fold higher content of indole-3-acetic acid (IAA) in the two poorly embryogenic genotypes in comparison to the highly reactive genotype 'A188'. Results were confirmed for wheat. Two cultivars which differed in their tissue culture ability were analysed for hormone levels comparing plants grown at 15°C and 25°C, respectively (Hess and Carman 1998). Embryogenic competence was associated with low IAA and abscisic acid (ABA) levels in seeds from 0 to 12 days post anthesis. Low growth temperatures delayed the rise in IAA and ABA thus increasing the embryogenic response. No detailed analyses were performed up to now on the influence of light intensity and especially spectral composition of light subjected to donor plants and *in vitro* response of explants. However, various studies have shown correlations between light and changes in auxin levels (reviewed in Tian and Reed 2001).

The composition of the culture medium is a further major parameter remarkably determining efficiency of cell and tissue culture systems independent of the explant used. A range of basal media have been repeatedly compared for the different cereal species and in most cases an MS-based medium (Murashige and Skoog 1962) was found superior to promote fast callus growth, development of embryogenic cultures and also regeneration capacity (Hanzel *et al.* 1985; Lührs and Lörz 1987; Redway *et al.* 1990; Bregitzer 1992). However, B_5 medium (Gamborg *et al.* 1968) also gave reliable results (Dale and Deambrogio 1979; Kott and Kasha 1984; Goldstein and Kronstad 1986; Kachhwaha and Kothari 1996).

The content and balance of plant growth regulators largely determine the *in vitro* response. Numerous detailed and extensive studies were undertaken to analyse a broad spectrum of auxins and cytokinins for culture initiation. In summary, 2,4-D is the most widely used growth regulator irrespective of the explant in all cereal species. Nevertheless, prolonged exposures of cell cultures to high concentrations of 2,4-D affects the frequency of regenerated plants and causes chromosomal abnormalities (Deambrogio and Dale 1980; Nabors *et al.* 1983; Ziauddin and Kasha 1990; Baillie *et al.* 1993). In contrast, Bregitzer *et al.* (1995) reported a positive relation between green plant regeneration and 2,4-D concentration. However, other auxins were re-evaluated to replace 2,4-D. Investigations in wheat and barley showed that 3,6-dichloro-*O*-anisic acid (dicamba) was more suitable for somatic embryogenesis induction and exhibited a higher frequency of regenerants than did 2,4-D (Hunsinger and Schauz 1987; Carman *et al.* 1987; Castillo *et al.* 1998; Trifonova *et al.* 2001; Mendoza and Kaeppler 2002; Przetakiewicz *et al.* 2003; Halámková *et al.* 2004). A comparison of 2,4-D and 4-amino-3,5,6-trichloropicolinic acid (picloram) revealed no differences for induction of embryogenesis from inflorescences of wheat, barley and tritordeum (*Hordeum chilense* × *Triticum turgidum* conv *durum*) (Barro *et al.* 1999). Surprisingly, regeneration from cultures derived from the picloram-containing medium was almost twice as efficient as regeneration from cultures induced on 2,4-D. A significant increase in the frequency of plant regeneration caused by picloram was also confirmed for embryo-derived cultures (Kachhwaha *et al.* 1997; Akula *et al.* 1999; He and Lazzeri 2001; Mendoza and Kaeppler 2002; Sharma *et al.* 2004). Additionally, data were provided on the interaction between type of auxin and genotype. The best response of seven out of eight barley cultivars evaluated was observed on dicamba with 2,4-D or dicamba alone whereas one cultivar gave the highest reaction on picloram (Przetakiewicz *et al.* 2003). A few studies indicated that 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is a better auxin source concerning callus proliferation (Lazar *et al.* 1983) and frequency of root and shoot forming cultures (Jelaska *et al.* 1884). Nevertheless, more than 7.83 µM (2.0 mg/l) of 2,4,5-T induced dramatic increases of sister chromatid exchanges in wheat callus (Murata 1989). Furthermore, an evaluation of the growth regulators 2,4-D and 2,4.5-T at five different concentrations in a range from 0.5 mg/l to 10.0 mg/l revealed altogether embryogenic callus formation but no regenerants were obtained from any of the 2,4,5-T treatments (Baillie *et al.* 1993).

With respect to the influence of cytokinins, inhibitory (Dale and Deambrogio 1979; Bayliss and Dunn 1979; Thomas and Scott 1985; Chang *et al.* 2003) as well as enhancing effects (Lazar *et al*. 1983; Rengel and Jelaska 1986; Carman *et al.* 1987; Kachhwaha and Kothari 1996; Cho *et al.* 1998; Jiang *et al.* 1998; Dahleen and Bregitzer 2002) on establishment and maintenance of regenerable cultures were described.

Maturation of somatic embryos and plant regeneration was promoted after transfer of embryogenic callus to medium with reduced auxin concentration. Cytokinins can improve plant regeneration (Gosch-Wackerle *et al.* 1979; Powell *et al.* 1983; Lührs and Lörz 1987; Barro *et al.* 1999; Cho *et al.* 1998; He and Lazzeri 2001; Chang *et al.* 2003), but there are also reports that a medium without growth regulators was sufficient (Cheng and Smith 1975; Weigel and Hughes 1985; Ahloowalia 1987; Bartók and Sági 1990; Ruíz *et al.* 1992; Kachhwaha *et al.* 1997; Varshney and Altpeter 2001).

Type and concentration of carbon source also determine efficiency of embryogenic callus formation. The carbohydrates serve not only as an energy supply, they also influence osmolarity of the culture medium. The importance of an increased osmotic value for reduction of germination of immature as well as mature embryos, for improvement of embryogenic callus formation and for maintenance of long-term embryogenic capacity was demonstrated for maize, wheat, rice and barley using sucrose, sorbitol, mannitol and maltose as well as salts and polyethylene glycol (Lu *et al.* 1983; Galiba and Yamada 1988; Brown *et al.* 1989; Ryschka *et al.* 1991; Sharma *et al.* 2005b). Moreover, an interaction of the carbohydrate and the type of auxin used was observed for wheat. In a detailed study the effects of four auxins and the effect of maltose versus sucrose were evaluated (Mendoza and Kaeppler 2002). The substitution of sucrose by maltose resulted in an increase of regeneration frequency from cultures derived from media containing 2,4-D and picloram whereas a decreased regeneration was found on media supplemented with dicamba.

Different organic supplements were investigated to improve the initiation of embryogenic cultures. Thus, the addition of 0.25 to 1.0 g/l casein hydrolysate (Lührs and Lörz 1987; Bregitzer 1992), 500 mg/l glutamine (Redway *et al.* 1990) or 0.2 mM L-tryptophan (Carman *et al.* 1987) were found to increased the induction of embryogenic cell cultures. Moreover, addition of 10 mg/l proline helped to maintain morphogenic competence of callus induced from immature wheat embryos for over two years (Kothari and

Table 2 Approaches used for transformation with regeneration[1] of *Hordeum vulgare* L.

Explant source[2]	Cultivar[3]	Method of transformation	Genes[4]	Reference
IE	Golden Promise	particle gun	*bar*, *uidA*, BYDVcp	Wan and Lemaux 1994
MS	Igri	particle gun	*bar*, *uidA*	Jähne *et al.* 1994
IE	Kymppi	particle gun	*nptII*	Ritala *et al.* 1994
PP	Igri	PEG	*nptII*	Funatsuki *et al.* 1995
IE	Golden Promise, Haruna Nijo, Dissa	particle gun	*hpt*, *uidA*	Hagio *et al.* 1995
PP	Kymppi	electroporation	*nptII*	Salmenkallio-Marttila *et al.* 1995
IE	Golden Promise	particle gun	(1,3-1,4)-β-glucanase, *bar*, *uidA*	Jensen *et al.* 1996
IE	Golden Promise, Femina, Salome, Corniche	particle gun	*bar*, *uidA*	Koprek *et al.* 1996
IE	Golden Promise	*Agrobacterium*	*bar*, *uidA*	Tingay *et al.* 1997
IE, EC	Golden Promise, Harrington, Galena	particle gun	*bar*, *uidA*	Cho *et al.* 1998
PP	Golden Promise	PEG	*nptII*	Kihara *et al.* 1998
MS	Igri	particle gun	*Vst1*, *bar*	Leckband and Lörz 1998
IE	Golden Promise, Baronesse	particle gun	*gfp*	Ahlandsberg *et al.* 1999
IE	Golden Promise	particle gun	*lysC*	Brinch-Pedersen *et al.* 1999
IE	Golden Promise	particle gun	*wtrxh*, *bar*	Cho *et al.* 1999a
IE	Golden Promise	particle gun	*bar*, *uidA*	Cho *et al.* 1999b
IE	Golden Promise	particle gun	*codA*, *P450gene*, *bar*	Koprek *et al.* 1999
IE	Golden Promise, Kymppi	particle gun	*egll*, *bar*	Nuutila *et al.* 1999
SMC	Harrington	particle gun	*bar*, *uidA*, *nptII*	Zhang *et al.*1999
IE	Golden Promise	particle gun	*bar*, *uidA*, *luc*	Harwood *et al.* 2000
PP	Igri, Alexis	microinjection	*uidA*	Holm *et al.* 2000
IE	Golden Promise	*Agrobacterium*	(1,3-1,4)-β-glucanase, *bar*	Horvath *et al.* 2000
PP	Igri	PEG	β-amylase, *nptII*	Kihara *et al.* 2000
IE	Golden Promise	particle gun	*Ac* TPase, *Ds* element	Koprek *et al.* 2000
PP	Clipper	PEG	*nptII*, *uidA*	Nobre *et al.* 2000
IE	Golden Promise	*Agrobacterium*	*xynA*, *bar*	Patel *et al.* 2000
IE	Golden Promise	*Agrobacterium*	BYDV-PAV hpRNA, *hpt*	Wang *et al.* 2000
MS	Igri	particle gun	*uidA*, *gfp*	Carlson *et al.* 2001
IE	Golden Promise	*Agrobacterium*	*LPI*, *AG*, *HPI*, *hpt*	Matthews *et al.* 2001
IE, MS	Golden Promise, Igri	particle gun	AcΔ102, *pat*	Scholz *et al.* 2001
IE	Golden Promise	*Agrobacterium*	*bar*, *uidA*	Trifonova *et al.* 2001
EC	Schooner	*Agrobacterium*	*PAVpol*, *RPVpol*, *bar*, *hpt*	Wang *et al.* 2001
IE	Golden Promise	particle gun	*SnRK1*, *bar*, *uidA*	Zhang *et al.* 2001
IE	Golden Promise	particle gun	*gfp*, *bar*	Cho *et al.* 2002
IE	Golden Promise	*Agrobacterium*	*gfp*, *hpt*	Fang *et al.* 2002
IE	Golden Promise	particle gun	*bar*, *luc*	Harwood *et al*. 2002
EC	Conlon	particle gun	*bar*, *uidA*	Manoharan and Dahleen 2002
IE	Golden Promise	*Agrobacterium*	*Amy-gfp-amy3´*, *hpt*	Matthews et al. 2002
IE	Golden Promise	particle gun	P1-MAR[5], TBS-MAR, *uidA*	Petersen *et al.* 2002
IE	Golden Promise	*Agrobacterium*	HIVDR	Schünmann *et al.* 2002
IE	Golden Promise	*Agrobacterium*	five human genes[6]	Stahl *et al.* 2002
EC	Golden Promise	*Agrobacterium*	*1033-asi::gfp*, *hpt*	Furtado *et al.* 2003
IE	Golden Promise	*Agrobacterium*	*Rpg1*, *bar*	Horvath *et al.* 2003
IE	Golden Promise	*Agrobacterium*	*HvGAMYB:gfp*	Murray *et al.* 2003
IE	Golden Promise	particle gun	*alkBA*, *bar*	Tull *et al.* 2003
IE	Golden Promise	*Agrobacterium*	*cel-hyb1*, *hpt*	Xue *et al.* 2003
IE	Golden Promise	particle gun	*Glu-1D-1:;* γ zein, *bar*, *uidA*	Zhang *et al.* 2003
IE	Golden Promise	particle gun	*SCBV::uidA*, *bar*	Al-Saady *et al.* 2004
IE	Golden Promise	*Agrobacterium*	*wt-Rp1D*, *hpt*	Ayliffe *et al.* 2004
IE	Golden Promise	*Agrobacterium*	*Mla1* and *Mla6* derivatives	Bieri *et al*. 2004
IE	Golden Promise, Schoner, Chebec, Sloop	*Agrobacterium*	*hpt*, *gfp*, *uidA*	Murray *et al.* 2004
IE	Golden Promise	*Agrobacterium*	*HORvu;Pht1;1*	Rae *et al.* 2004
IE	Golden Promise	*Agrobacterium*	*AtZIP1*, *hpt*	Ramesh *et al.* 2004
IE	Golden Promise	particle gun	*LDI*, *bar*	Stahl *et al.* 2004
IE	Golden Promise	*Agrobacterium*	*hpt*, *uidA*, *gfp*	Coronado *et al.* 2005
EC	Golden Promise	*Agrobacterium*	*EM::gfp*, *hpt*	Furtado and Henry 2005
IE	Golden Promise	*Agrobacterium*	*rach-G15V*, *gfp*, *hpt*	Schultheiss *et al.* 2005
MS	Igri	*Agrobacterium*	*Hv-elF4E*	Stein *et al.* 2005
IE	Golden Promise	particle gun, *Agrobacterium*	*bar*, *luc*, *uidA*	Travella *et al.* 2005
IE	Golden Promise	particle gun	*Lem2::gfp*	Abebe *et al.* 2006
ovules	Golden Promise	*Agrobacterium*	*hpt*, *gfp*	Holme *et al.* 2006
IE	Golden Promise	particle gun	*FaeG*, *bar*	Joensuu *et al.* 2006
MS	Igri	*Agrobacterium*	*bar*, *uidA*, *pat*, *hpt*, *gfp*	Kumlehn *et al.* 2006
IE	Golden Promise	*Agrobacterium*	*hpt*, *gfp*	Lange *et al.* 2006
IE	Conlon	particle gun	*Tri101*, *bar*, *uidA*	Manoharan *et al.* 2006
IE	Golden Promise	*Agrobacterium*	*Jekyll*, *gfp*	Radchuk *et al.* 2006
IE	Salome	particle gun	*LOX2:Hv:1*, *bar*	Sharma *et al.* 2006
IE	Golden Promise	*Agrobacterium*	*UbiDs*, *bar*, *uidA*	Ayliffe *et al.* 2007
IE	Golden Promise	*Agrobacterium*	*bar*, *uidA*, *hpt*, *gfp*	Shrawat *et al.* 2007
IE	Conlon	particle gun	*chi11*, *tlp*, *bar*	Tobias *et al.* 2007
EC	Dooweonchapssalbori, Igri	particle gun	*AtNDPK2*	Um *et al.* 2007
IE	Golden Promise	particle gun	*vhb*, *bar*, *uidA*	Wilhelmson *et al.* 2007

Table 2 (Cont.)

[1] Only original papers were given in the Table. Reports dealing with further analyses of transgenic plants were not included.
[2] IE = immature embryos, MS = microspores, PP = protoplasts, EC = embryogenic callus derived from immature embryos, SMC = shoot meristematic cultures.
[3] From papers giving several cultivars in M&M, only the cultivars were listed from which the generation of transgenic plants is evidenced.
[4] *bar* = phosphinothricin acetyl transferase gene from *Streptomyces hygroscopicus*, *uidA* = β-glucuronidase gene from *E. coli*, BYDVcp gene = barley yellow dwarf virus coat protein, *nptII* = neomycin phosphotransferase gene, *hpt* = hygromycin B phosphotransferase gene, *Vst1* = stilbene synthase from *Vitis vinifera*, *gfp* = green fluorescent protein gene, *lysC* = lysine-threonine feedback insensitive form of aspartate kinase, *wtrxh* = wheat thioredoxin h gene, *codA* = cytosine deaminase, P450gene = cytochrome P450 mono-oxygenase gene, *egll* = fungal thermotolerant endo-1,4-β glucanase, *luc* = firefly luciferase gene, *Ac* TPase gene = maize *Activator* transposase gene, *Ds* element = *Dissociation* inverted repeat ends, *xynA* = xylanase gene from *Neocallimastix patriciarum*, BYDV-PAV hpRNA = hairpin(hp)RNA containing sequences of barley yellow dwarf virus-PAV, *LPI* = low-pI α-amylase gene, *AG* = α-glucosidase gene, *HPI* = high-pI α-amylase gene, AcΔ102 = maize autonomous element *Ac (Activator), pat* = phosphinothricin acetyl transferase gene from *Streptomyces viridochromogenes*, *PAVpol* = polymerase coding region from barley yellow dwarf virus-PAV, *RPVpol* = polymerase coding region from cereal yellow dwarf virus-RPV, *SnRK1* = sucrose non-fermenting-1-related protein kinase, *Amy-gfp-amy3′* = gfp between promoter and terminator elements of barley high-pI α-amylase gene, HIVDR = HIV diagnostic reagent, *asi* = bifunctional α-amylase/subtilisin inhibitor, *Rpg1* = gene for resistance to stem rust caused by *Puccinia graminis* f. sp. *tritici*, *HvGAMYB* = MYB transcription factor from barley aleurone cells, *alkBA* = alkalophilic *Bacillus* α-amylase, *cel-hyb1* = hybrid cellulase gene, *Glu-1D-1* = high-molecular-weight glutenin subunit gene promoter, γ zein = γ zein protein of maize, *SCBV* = sugarcane bacilliform virus promoter, *wt-Rp1D* = maize gene which confers race specific resistance against *Puccinia sorghi*, *Mla* = allelic race specific resistance genes to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, *HORvu;Pht1;1* = barley high affinity phosphate transporter, *AtZIP1* = *Arabidopsis thaliana* zinc transporter, *LDI* = limit dextrinase inhibitor gene, *EM* = early methionine proteins, *rach-G15V* = mutant from RACB protein, *Hv-elF4E* = eukaryotic translation initiation factor 4E', *Lem2* = lectin-like protein of barley, *FaeG* = fimbrial adhesin gene, *Tri101* = gene for 3-OH trichothecene acetyltransferase, *Jekyll* = gene expressed in nurse tissue of barley, *LOX2:Hv:1* = lipoxygenase 2 from barley, *UbiDs* = *Ds* element containing two maize polyubiquitin promoters; *chi11* = rice chitinase gene, *tlp* = rice thaumatin-like protein, *AtNDPK2* = *Arabidopsis* nucleoside diphosphate kinase 2, *vhb* = *Vitreoscilla* haemoglobin
[5] *uidA* reporter gene flanked by matrix attachment regions (MAR) from soybean (P1) or petunia (TBS)
[6] antithrombin III, α-antitrypsin, serum albumin, lysozyme, lactoferrin

Varshney 1998). Besides that, an optimisation in media nitrogen composition due to an altered nitrate:ammonia ratio resulted in a significant improvement of regeneration (Nuutila *et al.* 2000; Chauhan and Kothari 2004).

Another variable studied was the concentration of micro-nutrients in the widely used MS-medium developed for tobacco tissue cultures. Adding a 50- to 500-fold higher concentration of cupric sulfate than in the original MS-medium to the callus induction, maintenance and regeneration media dramatically increased shoot regeneration from wheat, triticale (×*Triticosecale* Wittmack) and barley (Purnhauser and Gyulai 1993; Dahleen 1995; Bregitzer *et al.* 1998; Nuutila *et al.* 2000). However, different genotypes required special concentrations for optimal response (Dahleen 1995). Additionally, the incorporation of silver nitrate, an inhibitor of ethylen, was found to effectively increase shoot formation in wheat and maize tissue cultures (Purnhauser *et al.* 1987; Songstad *et al.* 1988). Recently, evidences were provided on the correlation between regeneration and ethylene evolution also for barley. Analysing ten-week old callus cultures from six barley cultivars, the production of this gaseous plant hormone was found to be highest from the high regenerable cultivar 'Golden Promise' and poorest from 'Morex' and 'DH-20' displaying lowest regenerability (Jha *et al.* 2007). Manipulation of ethylene production by addition of the ethylene precursor l-aminocyclopropane l-carboxylic acid or by the ethylene antagonist silver nitrate resulted in an improvement of green plant regeneration. However, timing of ethylene action seems to be critical for maximum regeneration in specfic barley genotypes (Jha *et al.* 2007).

In summary, during more than fifty years of cereal tissue culture extensive and detailed studies also in very recent time revealed various factors dramatically influencing morphogenic competence. A lot of data accumulated on the interaction of these parameters suggested that the development and use of genotype specific protocols by systematic testing of a range of key variables can enhance plant regeneration (Walmsley *et al.* 1995; Bregitzer *et al.* 1998). Nevertheless, despite the described significant developments in *in vitro* culture and manipulation of cereals recovery of transgenic plants is limited to a few species especially in case of barley (**Table 2**) and wheat.

THE IMPACT OF TDZ IN CEREAL TISSUE CULTURE

The plant growth regulator *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron, TDZ) originally registered as a cotton defoliant (Arndt *et al.* 1976) in 1976 by Schering AG (Berlin, Germany) was first described to have cytokinin activity in 1982. In comparison to the adenine-type cytokinins like benzylaminopurine (BAP), kinetin (Kin) or zeatin (Zea), this substance, a substituted phenylurea, does not contain the purine ring. TDZ promoted growth of cytokinin dependent callus cultures of lima bean (*Phaseolus lunatus*) and revealed the highest cytokinin activity out of nine thiadiazolylurea and 4-pyridylurea-derivatives compared (Mok *et al.* 1982). Furthermore, activity of TDZ was demonstrated in the traditional cytokinin-dependent bioassays of soybean (*Glycine max*) callus growth, radish (*Raphanus sativus*) cotyledon expansion and tobacco plantlet regeneration (Thomas and Katterman 1986). Based on these observations, the chemical was evaluated in many tissue culture studies revealing that TDZ is a powerful regulator of various morphogenic processes. By applying TDZ, a diverse set of reactions in tissue culture was described including callus induction, initiation of somatic embryos, adventitious shoot formation and axillary shoot proliferation. Consequently, this highly active cytokinin has received more and more attention as summarized in a survey of published reports demonstrating a dramatic rise in the number of publications in the second half of the nineties (Murthy *et al.* 1998) which is constantly increasing. Improved regeneration protocols were developed in a number of dicot species (Lu 1993). However, most impressive was the facilitation of efficient micropropagation of many recalcitrant woody species. According to Huetteman and Preece (1993) there is substantial evidence that TDZ is perhaps the most potent cytokinin for *in vitro* establishment and proliferation of difficult to propagate woody species. Besides that, TDZ showed a remarkable regenerative ability in leguminous plants also known to be recalcitrant (Lakshmanan and Taji 2000). In contrast, information available on the use of TDZ for *in vitro* culture of monocot species, especially for cereals and grasses, has been limited until recently.

Induction of shoot formation from callus cultures

TDZ was included in a study concerning improvement of plant regeneration from indica rice varieties (Tian *et al.* 1994). The results shown in this report demonstrated, that involvement of TDZ in combination with the auxins 2,4-D and α-naphthaleneacetic acid (NAA) in the callus induction medium enhanced shoot formation more than threefold in comparison to the cytokinins Zea, BAP, Kin and 6-γ-γ-(dimethylallylamino)-purine (2iP). Moreover, further analyses revealed that using TDZ alone in the subculture medium favoured plant regeneration more than TDZ combined with the auxins (Tian *et al.* 1994). Improved regeneration frequencies were also described for Australian rice varieties applying 5.37 µM (1 mg/l) NAA and 2.27-9.08 µM (0.5-2 mg/l) TDZ in comparison to BAP for mature embryo-derived callus. Nonetheless, the cultivars showed a varied response regarding number of shoots per regenerating callus depending on the cytokinin used (Azria and Bhalla 2000).

For wheat, several auxin/cytokinin combinations were assayed to promote shoot formation from callus derived

from immature embryos. The addition of TDZ as the sole growth regulator was superior to all other phytohormone variations concerning percentage of calli forming buds (Yu *et al.* 1999). Furthermore, the compound was evaluated in a detailed study including barley and wheat. Callus derived from immature embryos was analysed using the model genotypes 'Golden Promise' and 'Bobwhite' (Shan *et al.* 2000). TDZ concentrations ranging from 0.045-45.41 µM (0.01-10 mg/l) were examined. For both species, plant regeneration was observed at all levels of TDZ tested with the highest mean percent regeneration using 4.54 µM (1 mg/l) for barley and 0.91 µM (0.2 mg/l) for wheat. The optimum found was compared with other commonly used plant growth regulator formulations for barley and wheat, respectively. For both species, the TDZ-containing medium produced significantly higher mean percent regeneration than the often used hormone combinations comprising low 2,4-D and BAP or IAA and Kin (Shan *et al.* 2000). The promoting effect of TDZ on regeneration of wheat callus induced from immature as well as mature embryos in comparison to other cytokinins was confirmed for several Chinese spring wheat cultivars (Li *et al.* 2003). Very recently, a comparative analysis of morphogenic response of several explants including immature and mature embryos and immature inflorescences documented that addition of TDZ increased regeneration frequency significantly also for nine popular Indian wheat varieties from *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum* (Chauhan *et al.* 2007). Additionally, for embryogenic callus derived from mature barley embryos the formation of shoot buds with subsequent development of small shoots was favoured due to replacement of BAP by TDZ (Sharma *et al.* 2005b). Conversely, in a study to explore the *in vitro* regeneration from different explants of four commercially important barley genotypes from Western Canada, TDZ treatment did not stimulate plant formation from calluses derived from scutella and from leaf-bases/apical meristems, whereas addition of BAP induced shoot production (Ganeshan *et al.* 2003).

TDZ was also successfully used in sugarcane (*Saccharum* spp. hybrids) tissue culture. Embryogenic callus derived from immature inflorescences was subcultured on media containing different growth regulators. All TDZ treatments resulted in faster shoot regeneration than the Kin/NAA treatment, and more shoot production than either the 2,4-D or Kin/NAA treatments (Gallo-Meagher *et al.* 2000). In case of callus derived from young sugarcane leaves, five concentrations of five different cytokinins were examined. Medium containing TDZ yielded the highest percentage of shoot induction and the largest number of shoots with the optimal concentration of 2.5 µM (0.55 mg/l). Besides that, the utility of TDZ in comparison to 2,4-D and picloram for callus induction and plant regeneration from sugarcane seed explants was studied. No callus was obtained using TDZ alone, only shoot initiation at a high frequency was observed (Chengalrayan *et al.* 2005). These results correspond to the findings of Bai and Qu (2001) in an effort to optimise tissue culture response of turf-type tall fescue (*Festuca arundinacea*). TDZ was shown to improve callus regeneration frequency of mature seeds but did not promote callus induction suggesting that an auxin is necessary for callus induction. Moreover, the effect of TDZ on embryogenic cultures induced from caryopsis of kodo millet (*Paspalum scrobiculatum*) was studied revealing, that a combination of TDZ and the auxin 2,4-D stimulated the differentiation of shoot buds (Rashid 2002). The data demonstrate that frequency of differentiating shoot buds depends on the TDZ concentration.

Somatic embryogenesis

Various investigations in a wide variety of plants, including herbaceous as well as woody species, documented the high efficiency of TDZ in stimulating somatic embryogenesis (Huetteman and Preece 1993; Murthy *et al.* 1998; Lakshmanan and Taji 2000). Comparative analysis of BAP, Kin, 2iP and TDZ in combination with 2,4-D showed that TDZ was superior to the other cytokinins in numerous species (Lu 1993). A primary requirement for initiation of embryoids from somatic cells is the application of exogenous auxins or an alteration of the cytokinin/auxin ratio. Nevertheless, TDZ alone has been observed to induce somatic embryogenesis thus substitute for the requirement of both phytohormone classes (Murthy *et al.* 1998). These findings suggested a role of TDZ in modulation of auxin metabolism. Evidences were provided by Murch and Saxena (2001) using radiolabelled versions of TDZ. Their results indicate that TDZ-exposure enhances the accumulation and translocation of the auxin IAA within the tissues.

Surprisingly, reports on stimulation of somatic embryogenesis by TDZ in the *Poaceae* are rare up to now. Rashid (2002) described a variety of experimental approaches to establish an *in vitro* regeneration system from mature caryopsis of kodo millet (*Paspalum scrobiculatum*). Callus initiated on medium supplemented with 4.52-22.62 µM (1-5 mg/l) 2,4-D turned embryogenic within 6 weeks and differentiated into somatic embryos. However, the combined use of 4.52 µM 2,4-D and 4.54-22.72 µM (1-5 mg/l) TDZ resulted in shoot bud differentiation on the callus raised from the basal part of the seedlings whereas somatic embryos along with shoot buds were visible on other parts of the compact callus. No somatic embryos were observed at a combination of 11.25-22.72 µM (2.5-5 mg/l) TDZ along with 4.52 µM 2,4-D (Rashid 2002).

Contrasting observations were provided for caryopsis of indica rice cv. 'Jaumala' which formed a slow-growing non-morphogenic callus on 2,4-D–containing medium. A short three day exposure to 2,4-D followed by subculture on medium with 10 µM (2.2 mg/l) TDZ resulted in formation of compact callus which proliferated, turned green and differentiated to form somatic embryos regenerating into shoots. Somatic embryogenesis was also observed applying equimolar amounts of BAP but with a lower frequency (Gairi and Rashid 2004). In contrast, Sharma *et al.* (2005b), analysing the effect of cytokinins on primary callus induction from mature barley embryos, observed that very low doses of BAP (0.001 mg/l) enhanced embryogenesis whereas TDZ did not.

Multiple bud and shoot formation

Although TDZ was characterized to have powerful cytokinin-like activity resulting in a significant improvement of shoot multiplication in diverse recalcitrant species, the compound was introduced into cereal tissue culture for that purpose with a remarkable delay. In attempts to develop *in vitro* systems for switchgrass (*Panicum virgatum*), Gupta and Conger (1998) first described application of TDZ for differentiation of multiple shoots in the *Poaceae*. Using mature caryopsis as starting material, multiple shoot formation from the apical region and formation of a variable amount of callus at the mesocotyl part was observed due to application of different combinations of 2,4-D and TDZ. Replacement of TDZ with BAP failed to induce multiple shoots. Regeneration frequency and number of shoots per responding explant was significantly regulated by the ratio of 2,4-D and TDZ. The highest regeneration frequency as well as mean number of shoots per responding explant occurred on 4.52 µM (1 mg/l) 2,4-D and 18.2 µM (4 mg/l) TDZ in all the three genotypes evaluated. The results clearly showed that a rise in 2,4-D concentration decreased multiple shoot formation while an increase in TDZ concentration enhanced shoot formation. Interestingly, neither 2,4-D and TDZ alone initiated multiple shoots, and both substances were required (Gupta and Conger 1998). In contrast, analysing the effect of TDZ alone on mature caryopsis of kodo millet, Rashid (2002) observed a poor seedling development and a gradual degeneration of the main shoot followed by differentiation of multiple shoots from the base of the seedling. TDZ was favourable in a range of 4.54-22.72 µM (1-5 mg/l) reaching a maximum of 40% of the cultures which responded at

22.72 µM. BAP was found to be ineffective.

The utility of TDZ to induce multiple shoot formation also in small grain cereals was analysed in a variety of experimental approaches to establish *in vitro* regeneration protocols using (i) readily accessible explants and (ii) cultivars of high commercial value.

To overcome insufficient induction of embryogenic callus from mature embryos in barley, the explants were cultured directly on regeneration media containing low levels of 2,4-D in combination with BAP or TDZ (Ganeshan *et al.* 2003). Direct shoot production without a callus interphase was obtained yielding 1.5-3.5 shoots per explant due to the addition of 8.9 µM (2 mg/l) BAP and 3-8 shoots per explant in case of 4.54 µM (1 mg/l) TDZ. However, application of TDZ as the sole growth regulator at a concentration of 4.5 µM resulted in a further increase reaching 7.5-12 shoots per explant. In parallel, leaf-bases/apical meristems were examined as explants and responded favourably concerning multiple shoot production caused by TDZ (Ganeshan *et al.* 2003). The mature embryo system developed for barley was successfully reproduced for winter, spring and durum wheat as well as for oat and triticale. Direct multiple shoot production was reported yielding varying numbers of shoots per explant and requiring different optimal concentrations of TDZ depending on the species and the cultivar (Ganeshan *et al.* 2006a).

Furthermore, application of TDZ provided the basis for establishment of *in vitro* cultures containing small shoots and tight clusters of shoot buds with unexpanded leaves in a short period of 9-10 weeks, which were described for model and commercial barley cultivars (Sharma *et al.* 2004). Using meristematic shoot segments excised from germinated mature embryos thirty eight combinations and concentrations of four different auxins and two cytokinins were analysed revealing that the combined use of 8.28 µM (2 mg/l) picloram and 13.62 µM (3 mg/l) TDZ favoured highest formation of multiple shoots and additional buds already after 3 weeks. These responses were strongly determined by culture of mature embryos directly on the appropriate hormone formulation with the axis side down to avoid continued elongation of the main shoot. The meristematic shoot segments were prepared after 5-7 days of culture and further cultured on the same medium. Three weeks later, many multiple shoots with a minimum number of 10 per explant and a maximum length of 2-3 cm were formed. Moreover, many new axillary buds were obtained. Shoots with a length of 2-3 cm were cut and the clumps recultured or divided into sectors and transferred onto medium containing 8.28 µM (2 mg/l) picloram and 11.35 µM (2.5 mg/l) TDZ. Vigorously growing shoots with a maximum up to 2 cm in length along with many shoot buds were found after 3 weeks. The expanded leaves were removed again and clumps with remaining small shoots, less than 1 cm in length, and shoot buds (clusters of the meristematic domes with unexpanded leaves) were subdivided again and cultured on the same medium. Elongation of the main shoot was not observed. Enrichment of buds was achieved due to biweekly subcultures by cutting elongated leaves of the multiple shoots and subdivided the clumps repeatedly, whereas shoot elongation of the multiple shoots became rare in subsequent passages. These cultures were obtained after 8 to 9 weeks and could be kept in a maintenance and proliferation stage for over 6 months (Sharma *et al.* 2004). Shoot development and elongation could be induced by supplement of 0.41 µM (0.1 mg/l) picloram and 4.54 µM (1 mg/l TDZ). After 2 to 3 weeks a maximum elongation up to 6 cm was scored and strengthened shoot growth was promoted by a 2-3 week culture on medium devoid of hormones. The shoots were rooted on basal medium with 4.9 µM (1 mg/l) indole-3-butyric acid (IBA), transferred into soil and developed normally (Sharma *et al.* 2004). Clumps containing high regenerative green tissue were established from all the cultivars included in the study after 9-11 weeks and gave rise to an average shoot number of 21-27 demonstrating, that the procedure is less genotype dependent.

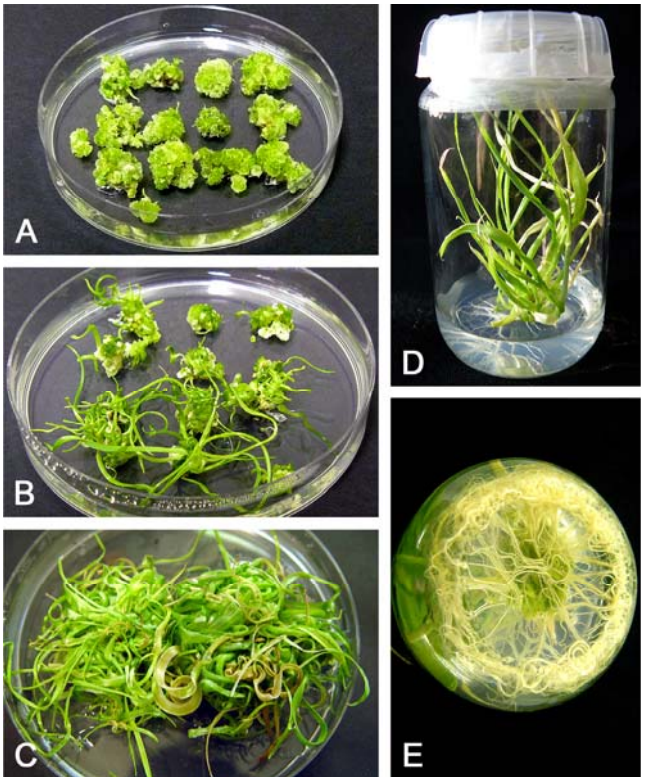

Fig. 1 Morphogenic response of four year old cultures established from meristematic shoot segments from barley and wheat as described by Sharma *et al*. (2004; 2005c). (**A**) Green clusters of shoot buds on maintenance and proliferation medium containing 2 mg/l picloram and 2.5 mg/l TDZ, *H. vulgare* cv. 'Merlot'. (**B**) Redifferentiation of shoots from bud clumps two weeks after transfer on regeneration medium containing 0.1 mg/l picloram and 1 mg/l TDZ, *T. aestivum* cv. 'Dekan'. (**C**) Multiple shoot formation and elongation after 6 weeks of culture on regeneration medium *T. aestivum* cv. 'Dekan'. (**D**) Rooting of shoots on medium with 1 mg/l IBA and (**E**) formation of a well developed rooting system after eight weeks of culture in barley cv. 'Lomerit'.

This simple and highly efficient short-term *in vitro* regeneration system was also suitable for wheat. Two comercially used European winter wheat cultivars 'Dekan' and 'Drifter' gave a high response concerning percentage of meristematic shoot segments forming multiple shoots as well as average shoot number per responding explant ranging between 28 and 33 shoots after 10-12 weeks. Almost no differences were found between both cultivars thus indicating a very low genotype dependency when meristematic shoot segments were used as an alternative explant source and cultures of multiple shoot clumps due to application of TDZ and picloram (Sharma *et al.* 2005c). Interestingly, cv. 'Dekan' was also included in a genotype screening of 38 European winter wheats using immature embryos (Varshney and Altpeter 2001) revealing a poor response ranking at position 35 for regenerated shoots per embryo, thus emphasizing the advantage of the method provided by Sharma *et al.* (2004, 2005c). Surprisingly, the morphogenic potential of these cultures is maintained for over 4 years now by regular subcultures for barley as well for wheat (**Fig. 1A-D**).

The feasibility of this high potent culture system was studied for nodes from barley and wheat (Sharma *et al.* 2007). The combination of 8.28 µM (2.0 mg/l) picloram and 4.54-22.72 µm (1-5 mg/l) TDZ favoured induction of clumps of multiple shoots and buds with or without callus formation from nodal explants excised from *in vitro* grown plants. Within 8-10 weeks upon further subcultures, the proliferation into little callus with rapidly and continuously forming adventitious buds containing clusters of meristemoids, termed meristematic bulk tissue was obtained. The proliferation of shoot buds can be maintained for long-term at the meristemoid stage without promoting of shoots (inhib-

iting shoot differentiation) on media containing higher levels of picloram (8.28-12.42 µM) and TDZ (9.08-13.62 µM). Shoot development can be achieved by lowering the levels (0.45 µM) of both the hormones promoting shoot differentiation. Highly morphogenic cultures using nodal shoot segments from multiple model and elite cultivars in barley and wheat were established with a frequency ranging between 37% and 82% (Sharma *et al.* 2007). The organogenic competence of nodes raised from *in vitro* and *ex vitro* grown plants repeatedly described for dicots for several decades was rarely demonstrated for cereals (Dale *et al.* 1981). This system further extends the kind of tissues suitable for *in vitro* culture and plant regeneration in cereals.

In all the studies describing multiple shoot and bud formation in cereals and grasses due to involvement of TDZ the compound was effective in a range of 4.54-22.72 µM (1-5 mg/l), which is relatively high in comparison to 10 nM-3 µM (0.0022 mg/l-0.66 mg/l) which is generally used for dicots (Lu 1993). In many recalcitrant woody species concentrations lower than 1 µM (0.22 mg/l) were shown to induce greater axillary proliferation than the other cytokinins (Huetteman and Preece 1993) suggesting different requirements for hormones for dicots and monocots with respect to their morphogenic response.

Mode of multiple shoot clump formation

The high morphogenic potential of shoot meristems was already documented in earlier reports. Using extremely immature shoot meristems from wheat, cultures capable of plant regeneration were successfully initiated. These cultures were kept in a proliferating budding state in the presence of 9.05 µM (2 mg/l) 2,4-D, whereas the removal of 2,4-D resulted in an outgrowth of shoots and roots (Wernicke and Milkovits 1986). In attempts to identify alternative and highly regenerative explant sources in comparison to the most commonly used immature embryos, the differentiation of multiple shoot clumps from shoot tips caused by the cytokinin BAP was first described for maize (Zhong *et al.* 1992). In the following years protocols for establishing shoot meristematic cultures have been successfully developed for oat (Zhang *et al.* 1996), barley (Zhang *et al.* 1998), sorghum (Zhong *et al.* 1998), pearl millet (Devi *et al.* 2000), finger millet (*Eleusine coracana*) (Kumar *et al.* 2001) and wheat (Ahmad *et al.* 2002). In all these reports excluding finger millet, shoot apices from aseptically germinated one week old seedlings were cultured on media with 2.26 µM (0.5 mg/l) 2,4-D and 8.9-17.8 µM (2-4 mg/l) BAP and differentiated multiple shoots with a high frequency. For finger millet, Kin was more effective than BAP. Biweekly subcultures resulted in the formation of multiple shoot clumps differentiating axillary and adventitious buds, which was clearly documented by scanning electron microscopy (Zhong *et al.* 1992; Zhang *et al.* 1996; Zhong *et al.* 1998).

Early morphological changes in development of meristems exposed to TDZ were studied in detail by histological analyses in combination with scanning electron microscopy (Gupta and Conger 1998). For switchgrass, five-day-old germinated seedlings showed normal development whereas the suppression of primary meristem growth and inhibition of internode elongation, accompanied by proliferation of axillary buds were observed as early as ten days after culture initiation. These changes led to induction of shoot meristems which developed in a nested array at the axil of the primary leaves. After another 2-3 days, differentiation of shoot meristems continued and leafy structures with trichomes formed. These newly emerged shoot meristems subsequently formed clumps of multiple shoots, which were detected in different developmental stages indicating an asynchronous mode of development. Histological studies evidenced the formation of numerous buds at the apical dome in clusters of multiple shoot clumps obtained after 21 days. Proliferation of shoot clusters was accompanied by formation of adventitious buds, which have no visible connection with the original vascular tissue suggesting *de novo* origin (Gupta and Conger 1998).

For wheat, direct multiple shoot induction from mature embryos in response to TDZ was also witnessed by histological analyses. Shoot formation obtained originate directly from the primary explant with no evidence of primary callus induction (Ganeshan *et al.* 2006a). Moreover, the tight clusters of multiple buds developed after 3-4 weeks of first subculture of nodes on TDZ-containing medium were characterized by reduced leaf primordia differentiation. Lateral sections revealed several meristemoids developing on the surface of proliferating nodular compact meristematic bulk tissue (Sharma *et al.* 2007).

The pattern of development of multiple shoot clumps caused by TDZ occurred in a manner very similar to that described for BAP-induced cultures. However, the time frame needed was shortened by TDZ. The data presented for application of BAP to establish meristematic shoot cultures outlined that clumps with adventitious shoot formation were obtained 12 weeks after culture initiation for maize, oat, sorghum, pearl millet and wheat (Zhong *et al.* 1992; Zhang *et al.* 1996; Zhong *et al.* 1998; Devi *et al.* 2000; Ahmad *et al.* 2002). For barley, the initial development of shoot meristematic cultures using BAP was described to be slow compared to the development of standard embryogenic callus derived from immature embryos but no exact time schedule was given (Zhang *et al.* 1999; Bregitzer *et al.* 2002). For genetic transformation 9-months-old shoot meristematic tissue was used which was induced on an improved medium (Zhang *et al.* 1999). However, the use of TDZ resulted in a shortage of time necessary to establish this type of highly differentiating cultures in commercial cultivars of barley since only eight weeks were needed (Sharma *et al.* 2004). For switchgrass and wheat, multiple shoot clumps were observed as early as three to four weeks after initiation (Gupta and Conger 1998; Sharma *et al.* 2005c).

Biochemical effects of TDZ exposure

Despite many investigations characterizing TDZ as a potent regulator of plant growth and development which induces cytokinin and auxin-type responses in tissue culture, the mode of action of the substance remains unclear. It is assumed that the high activity of TDZ is related to its stability in tissue culture. Using radiolabelled TDZ, the metabolism of the compound was analysed in callus of lima bean revealing that TDZ was not metabolized within the first 48 hours of culture and the primary metabolites were shown to be glucoside residues (Mok and Mok 1985). Consequently, the substance is not degradated by cytokinin oxidase (Mok *et al*. 1987). Moreover, a partially purified cytokinin oxidase from wheat has been shown to be strongly inhibited by diphenylurea (Galuszka *et al.* 2000). In addition, experiments were conducted using hypocotyl tissue of geranium (*Pelargonium* × *hortorum*) to determine the effect of TDZ exposure on auxin transport. The results demonstrate that TDZ functions as an intact molecule in both a free and conjugated or sequestered form. Furthermore, the functions of auxin accumulation and transport are maintained and enhanced in the tissues exposed to TDZ (Murch and Saxena 2001). Very recently, evidence was provided that the levels of IAA and endogenous indoleamines like melatonin and serotonin were enhanced in tissues showing regeneration caused by TDZ (Jones *et al.* 2007). Besides that, a decrease in TDZ-stimulated regeneration was found after application of an auxin-transport inhibitor and an auxin action inhibitor, respectively but concentration of melatonin and serotonin increased. Inhibitors of calcium and sodium transport also reduced the TDZ-induced regeneration. The authors suggested the TDZ-induced morphogenesis as a metabolic cascade, which includes an initial signal, accumulation and transport of endogenous plant signals and a system of secondary messengers (Jones *et al.* 2007).

Application of TDZ-based protocols

Various reports over the last decade have clearly evidenced that TDZ reveals activities exceeding that of other cytokinins also for the cereals and grasses. Its regulatory role of *in vitro* morphogenesis comprises shoot induction from callus as well as initiation and development of shoot meristematic clumps. The substance is highly efficient concerning enhancement of shoot formation from callus with respect to the (i) percentage of responding explants, (ii) the number of shoots per explant and (iii) the faster induction of shoots relative to previously published protocols. These features are of remarkable importance for application of biotechnological approaches for crop improvement. Protocols enabling a high, fast and reproducible regeneration of plants are urgently needed for genetic engineering since the stress caused by transformation independent of the method used as well as the stress caused by selection independent of the selectable agent applied dramatically reduce efficiency of transformation. Despite the vast amount of literature screening genotypes, medium composition and explants as presented for barley as an example (**Table 1**), successful transformation is limited to very few genotypes and the majority of all reports is concentrated to one or a few model genotypes (**Table 2**).

The results obtained by Shan *et al.* (2000) documented the superiority of the compound in comparison to commonly applied growth regulators since model genotypes in barley and wheat were used and a significant improvement of regeneration frequency was demonstrated. Besides that, Chauhan *et al.* (2007) very recently described the development of an efficient genotype independent *in vitro* regeneration system by manipulating the concentration and time of exposure to TDZ.

The suitability of the shoot meristematic cultures characterized by proliferation of tightly packed clusters of continuously multiplying axillary and adventitious buds has been shown to allow genetic transformation in maize, rice, barley, oat, sorghum and millet (for review see Sticklen and Oraby 2005). This easy-to-handle system can be established based on a low auxin/high cytokinin ratio using dry mature seeds being available without limit at any time. Furthermore, the protocols published document a low genotype dependency (Zhong *et al.* 1992; Zhang *et al.* 1996; Zhong *et al.* 1998; Ahmad *et al.* 2002; Ganeshan *et al.* 2003; Sharma *et al.* 2004, 2005c; Ganeshan *et al.* 2006a) thus allowing the application of gene transfer methods to varieties of agronomical value. With respect to the growth regulators used, the combination of 2,4-D and BAP first was described to be efficacious as summarized by Sticklen and Oraby (2005) and only in a few cases Kin yielded better responses. Nevertheless, the involvement of the high potent cytokinin-like substance TDZ extended the use of shoot apical meristems as an alternative explant to establish high regenerable cultures also for species for which BAP was not or only less effective (Gupta and Conger 1998; Rashid 2002) as well as for certain elite lines in barley and wheat known to be recalcitrant (Ganeshan *et al.* 2003; Sharma *et al.* 2004, 2005c; Ganeshan *et al.* 2006a). Additionally, TDZ offers the advantage of a very fast establishment of these cultures. Furthermore, evidences were provided that plants derived from shoot meristematic cultures reveal enhanced genomic stability relative to those regenerated from embryogenic callus since problems associated with methylation, albinism and mutations due to somaclonal variation were strongly reduced (Bregitzer *et al.* 2002). Very recently, by use of TDZ the establishment of a genotype-independent regeneration system for *indica* rice was reported which was shown to be suitable for *Agrobacterium*-mediated transformation and generation of T_0 was evidenced (Yookongkaew *et al.* 2007).

These high regenerable cultures can also be used for investigations regarding meristem development (Zhang and Lemaux 2004) and the involvement of plant growth regulators (Francis and Sorrell 2001). Besides that, functional analyses of meristem activity combined with studies concerning genetic control of cell proliferation as well as expression of cell cycle genes in shoot apical meristems are possible (Gegas and Doonan 2006). Thus, shoot meristematic cultures from maize and barley have been used to analyse the expression of KNOTTED1 (KN1), a protein necessary for maintenance of the shoot meristem. It was shown that KN1 or KN1-homologue(s) expression was retained in meristematic cells during *in vitro* proliferation of axillary shoot meristems (Zhang *et al*. 1998).

Long-term regeneration

One of the main problems in cereal tissue culture was the decline of regeneration potential after a short culture period (Vasil 1987; Lörz *et al.* 1988). The regenerative capacity of embryogenic callus was often lost after five to six subcultures with a very few exceptions describing long-term maintenance of morphogenesis (Nabors *et al.* 1983; Redway *et al.* 1990; Kachhwaha *et al.* 1997; Kothari and Varshney 1998). The loss of regenerability was observed for all commonly used explants like immature embryos (Gosch-Wackerle *et al.* 1979; Deambrogio and Dale 1980; Breiman 1985; Goldstein and Kronstad 1986; Chawla and Wenzel 1987; Lührs and Lörz 1987; Rotem-Abarbanell and Breiman 1989; Ziauddin and Kasha 1990; Bregitzer 1992; Baillie *et al.* 1993; Kachhwaha and Kothari 1994; Ganeshan *et al.* 2003), mature embryos (Rengel 1987), immature inflorescences (Rajyalakshmi *et al.* 1988), seedling mesocotyls (Jelaska *et al.* 1984) and leaf bases (Mohanty and Ghosh 1988). Since the transformation procedure requires several steps of selection regardless of the method used, the short-lived ability of cereal cell cultures dramatically reduces practical application of *in vitro* genetic manipulation.

The establishment of highly differentiating clusters containing multiple axillary and adventitious buds originated from shoot apical meristems or meristematic shoot segments provided an alternative regeneration system with low genotype dependency. The multiple shoot clumps can be maintained *in vitro* by regularly subcultures for long periods. The multiplication efficiency of these cultures was observed to remain consistent and high based on the use of BAP and 2,4-D for more than 18 months in the case of sorghum (Zhong *et al.* 1998) and over two years for maize (Sticklen and Oraby 2005). For wheat, long-term maintenance of morphogenic capacity of multiple shoot clumps was reported for more than 12 months due to the combined use of TDZ and picloram (Sharma *et al.* 2005c). Moreover, these cultures continue to multiply and regenerate normal green plants also after four years (data not published). In contrast, Wernicke and Milkovits (1986) who first demonstrated the high morphogenic competence of shoot meristems in the presence of 2,4-D, also followed proliferation of the cultures for a year. However, they described a remarkable decrease in the percentage of cultures able to regenerate shoots, an overgrowth by root-type tissues and an increase in the frequency of albino shoots. No data concerning longevity were given by Ahmad *et al.* (2002).

Ganeshan *et al.* (2003) who developed a mature embryo system for barley based on TDZ as the sole growth regulator found, that the number of subcultures led to a further increase in the total number of shoots produced from the mature embryos. They concluded that the system offers the possibility of establishing long-term shoot production cultures without loss of regeneration potential (Ganeshan *et al*. 2006a). Accordingly, the multiple bud/shoot clumps in barley and wheat raised from meristematic shoot segments (Sharma *et al.* 2004, 2005c) reveal long-term retention of morphogenecity for more than four years now (**Fig. 1**) and for the node-derived cultures (Sharma *et al.* 2007) two years were noted (data not published). In both systems, normal green plants regenerate after a reduction of the concentration of the phytohormones as described (Sharma *et al.* 2004, 2005c, 2007).

Problems associated with the use of TDZ

Despite the high cytokinin-like activity of TDZ for dicots, some analyses have shown disturbance of normal plant development associated with the use of that growth regulator. Such undesirable changes observed are hyperhydricity of the recovered shoots, abnormal leaf morphology like fasciated shoots, short and compact shoots and problems in elongation and rooting of the regenerated shoots as reviewed in Lu (1993) and Huetteman and Preece (1993).

In most of the studies evaluating TDZ for improvement of plant regeneration in the cereals and grasses, normal plant development without any visible abnormalities was described (Gallo-Meagher *et al.* 2000; Ganeshan *et al.* 2003; Sharma *et al.* 2004, 2005c; Ganeshan *et al*. 2006a; Chauhan *et al.* 2007; Sharma *et al.* 2007). However, in a few reports detrimental side effects of TDZ were also mentioned for cereals. For wheat, Li *et al.* (2003) reported that higher concentrations of TDZ tended to suppress root formation from shoots. In contrast, Shan *et al.* (2000) found no negative effect of TDZ on calli or regenerated plantlets neither from wheat nor from barley excluding little browning and necrosis of calli in barley on TDZ-containing regeneration medium. Besides that, no differences were obtained concerning plant morphology and seed set between plants regenerated from TDZ and those from other growth regulator combinations (Shan *et al.* 2000). Using TDZ for regeneration from embryogenic callus derived from young leaves of sugarcane, the substance produced the largest number of shoots. Nevertheless, it had the lowest percentage of shoots that were more than 1 cm in length (Chengalrayan and Gallo-Meagher 2001) confirming observations on reduced shoot length in dicots (Lu 1993; Huetteman and Preece 1993). Furthermore, an investigation aimed at improvement of plant regeneration from mature embryo-derived callus by TDZ in several rice varieties resulted in the regeneration of some albino shoots in every variety tested (Azria and Bhalla 2000). Altogether, the difficulties listed above were observed in cases in which plant regeneration was induced from callus implying the use of dedifferentiated tissues. In all the studies where plant development occurred from multiple bud and shoot clumps induced by TDZ, no problems with respect to shoot elongation, rooting, acclimatization, transfer into soil and seed set were described (Gupta and Conger 1998; Rashid 2002; Ganeshan *et al.* 2003; Sharma *et al.* 2004, 2005c, 2007). Moreover, plants regenerated from four-year-old cultures from elite lines in barley and wheat (Sharma *et al.* 2004, 2005c) continuously subcultured exhibit normal root development (**Fig. 1E**). These observations emphasize the use of shoot meristematic cultures as suggested by Bregitzer *et al.* (2002) and Sticklen and Oraby (2005).

Data on somaclonal variation induced by TDZ are not available for the cereals and grasses up to now. Moreover, a putative mutagenic influence of TDZ on long-term cultures remains to be analysed.

CONCLUDING REMARKS

Considerable progress has been made concerning *in vitro* regeneration of cereals and grasses during the last decades screening genotypes worldwide, various explant sources and numerous media constituents. In spite of these advances, the number of highly responsive genotypes suitable for genetic transformation experiments is still limited due to extensive genotypic variation for tissue culture performance. Interestingly, much data has accumulated during the last decade indicating that TDZ shows powerful cytokinin-like activity also for cereals and grasses in addition to the earlier reported high activity for recalcitrant dicots (Lu 1983; Murthy *et al.* 1998; Lakshmanan and Taji 2000). The results summarized above are very exciting since they offer the possibility to extend the range of genotypes amenable to biotechnological approaches to elite cultivars, which is highly relevant for the future improvement of these major crops.

TDZ was found to be superior in promoting morphogenic response from (i) callus induced from various explants, (ii) from a wide range of species and cultivars, (iii) reduces time necessary for establishment of regenerating cultures, (iv) enhances frequency of responding explants as well as the (v) number of shoots per explant. The substance can be used in cases, where other cytokinins are not or only less effective. Nevertheless, also for that high potent plant growth regulator conditions have to be optimised concerning combination with other hormones since several reports indicate highest organogenic capability, if TDZ is applied as the sole growth regulator while other studies describe the need to couple with a certain auxin. Moreover, despite the high efficacy of TDZ, also for that growth regulator genotype × medium interaction were reported thus an optimisation of concentration and time exposed to the substance is necessary. However, there are still some problems and uncertainties which have to be resolved. Thus, little is known so far about genetic stability and somaclonal variation especially after long-term exposure as well as agronomic performance of regenerated lines and further research is needed.

Finally, fifty years after the observation made by Skoog and Miller (1957) on manipulation of shoot and root development controlled by the ratio of auxins and cytokinins the assessment of TDZ as a potent regulator also for cereal tissue culture offers the possibility to further reduce recalcitrant nature of the *Poaceae*.

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