

Tolerance to Root Flooding of Wheat Plants (*Triticum aestivum* L.) Produced with Biotechnological Approaches

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ABSTRACT

Tolerance to soil flooding of wheat plants produced by *in vitro* selection of cells more tolerant to anoxia and regeneration of plants from such cells and transgenic wheat plants harboring the *ipt* gene encoding isopentenyltransferase, a key enzyme of cytokinin synthesis, was studied. On the basis of preliminary data, it was concluded that these biotechnological approaches are promising for improving tolerance to anaerobic stress of plants, which, like wheat, are highly sensitive to hypoxia and anoxia.

Keywords: anoxia, *in vitro* cell selection, transgenic plants

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; CaMV, *Cauliflower mosaic virus*; MS, Murashige and Skoog; NAA, naphthylacetic acid

INTRODUCTION

Waterlogged and submerged soils occupy vast territories in many countries (Maltby 1991). Plants inhabiting these lands, especially crops, suffer strongly from such extreme conditions (Musgrave and Ding 1998; Collaku and Harrison 2002). About 20% of areas used for wheat cultivation are subjected to annual flooding (Setter and Waters 2003). Although on waterlogged and submerged soils plants experience the action of a set of unfavorable factors, deficiency of oxygen (hypoxia) and especially its complete absence from the root zone (anoxia) are the main factors resulting in plant damage and even mass plant death (Vartapetian 1978; Kozłowski 1984; Jackson *et al.* 1991; Jackson and Black 1993; Visser *et al.* 2003). Nevertheless, many plant species, predominantly belonging to the wild flora, developed during their evolution properties facilitating to some degree their life not only on waterlogged but even on submerged soils (Armstrong *et al.* 1994; Jackson and Armstrong 1999; Colmer 2003; Vartapetian *et al.* 2008). Although some researchers believe that there are more than 20 mechanisms of plant adaptation to anaerobic stress (Setter *et al.* 2009) recent advances in the studying of causes for damaging and death of sensitive plants under conditions of hypoxia and anoxia and also the mechanisms of tolerant plant adaptation under such extreme conditions confirmed the concept about two main strategies of plant adaptation to anaerobic environment (Vartapetian 1978; Vartapetian and Jackson 1997; Jackson *et al.* 2009): **true** plant tolerance realized due to major rearrangement of plant metabolism under hypoxia or even complete oxygen absence (Loreti *et al.* 2003; Sachs and Vartapetian 2007; Ismail *et al.* 2009; Magneschi and Perata 2009) and **apparent** tolerance when plants inhabiting anaerobic medium avoid anaerobiosis due to oxygen transport from aerated plant parts to the organ located in the anaerobic environment (Armstrong *et al.* 1994; Jackson and Armstrong 1999; Colmer 2003). Available experimental data indicate that true tolerance of plant organs to hypoxia and anoxia is provided due to the control of mobilization and utilization of endogenous carbohydrate sources (Vartapetian and Jackson 1997; Sachs and Vartapetian 2007; Jackson *et al.* 2009; Magneschi and Perata 2009). On this

basis, the technology of *in vitro* selection of the callus cells tolerant to oxygen deficit has been elaborated (Stepanova *et al.* 2002). This technology is directed to the *in vitro* selection of the cells with changed energetic and carbohydrate metabolism. Earlier investigations performed on the sugarcane callus have shown that cell selection is a promising approach for production of plants tolerant to root flooding. In this work, we studied tolerance to root flooding of wheat plants produced by improved technology of cell selection as compared to that elaborated in experiments with sugarcane (Stepanova *et al.* 2002).

Another approach for improving wheat plant tolerance to root flooding that we also used in this study had a principally other basis. Cytokinin is known to retard plant senescence (Romanov 2009). Since under soil flooding clear signs of senescence and subsequent death are manifested in such sensitive to oxygen deficit plants as wheat (Trought and Drew 1980), we supposed that stimulation of cytokinin synthesis in transgenic wheat plants should delay to some degree their cell senescence and thus counteract the damaging effects of soil flooding. Therefore, the next stage of our work was the investigation of soil flooding tolerance of transgenic wheat plants harboring the *ipt* gene encoding isopentenyltransferase, a key enzyme of cytokinin synthesis, which have been produced earlier (Stepanova *et al.* 2006).

MATERIALS AND METHODS

Early-ripening cv. 'Enita' of spring soft wheat (*Triticum aestivum* L.) was used in experiments. Callus was initiated from immature embryos and cultured on the modified Murashige and Skoog (1962) (MS) nutrient medium supplemented with 20 g/l sucrose, 2.5 mg/l 2,4-D, and 10 mg/l AgNO₃. The cells were grown at 26 ± 1°C and a 16-h photoperiod (150 μmol photons m⁻² s⁻¹). Tolerant callus cells were selected after 32-h anaerobic incubation in modified carbohydrate-free MS medium (Stepanova *et al.* 2002). For plant regeneration, calluses were transferred to hormone-free MS medium. Plantlets produced were transferred onto half-strength MS and 1 mg/l NAA nutrient medium for rooting. Plants with well developed roots were planted in soil.

Transgenic wheat plants, which were also used in experiments with root flooding, were produced by *Agrobacterium*-mediated

Table 1 R₁ and control plants survival at different temperature regimes under condition of root flooding. Plants were grown under drained condition for 14 d and then treated under root flooding for 8 d or 10 d. Then plants were transferred to normal condition. Control plants were obtained from calli that did not pass through the selection procedure. R₁ plants were obtained from *in vitro* selected plants R₀. Root flooding was achieved by watering with tap water so that a 5-cm water layer was above the soil surface.

Treatment	Genotype	Total number of plants	Number of survived plants	% of survived plants
10 days of flooding, t° = 22°C	control plants	32	18	56
	R ₁ plants	36	36	100
8 days of flooding, t° = 32°C	control plants	20	0	0
	R ₁ plants	22	7	32

transformation and comprised the *ipt* gene under the 35S CaMV promoter and a selective *nptII* gene, encoding neomycin phosphotransferase determining tolerance to kanamycin, under the nopaline synthase promoter. Transformation was carried out as described earlier (Stepanova *et al.* 2006). In experiments on root flooding, progeny (T₁) produced by selfing of fertile transgenic (T₀) plants were used.

Plants obtained using *in vitro* cell selection and transgenic T₁ plants comprising the *nptII* and *ipt* genes were kept for 8, 10, 14 and 16 days under a 16-h photoperiod (150 μmol photons m⁻² s⁻¹) under conditions of soil flooding. Root flooding was achieved by watering with tap water so that a 5-cm water layer was above the soil surface. 10-36 plants were used in each treatment.

The effect of anaerobic stress was assessed by comparing control and experimental plant survival, growth and yield.

Data management and statistical analyses were performed using SPSS Statistics 11.0 software. Mean values were expressed with their standard deviations (SD) and compared by ANOVA using Least Square Difference (LSD) test and Student's T-test at the 0.05 significance level.

RESULTS

Tolerance to root flooding of wheat plants produced using *in vitro* cell selection

Plants regenerated from the calli, which had been subjected to oxygen deficiency for 32 h and control plants that did not pass through the selection procedure, were tested under conditions of soil flooding. After 16 days of soil flooding, only 33% of control plants vs. 73% of experimental plants survived.

For elucidation of genetic nature of selected cells and regenerated whole plants, the seeds collected from plants after cell selection were used for generation of progeny and the analysis of descendant tolerance to soil flooding. On the average, 50 seeds were taken from each plant. The progeny (R₁) produced by selfing of regenerants (R₀) were tested under soil flooding at different temperatures: 22 and 32°C (Table 1). It was demonstrated that, at both temperatures, the survival of descendants of selected plants was noticeable higher than of control plants (Fig. 1, Table 1). The distinctions were especially evident at higher temperature (32°C).

Tolerance to root flooding of *ipt*-transgenic wheat plants

Transgenic T₀ plants harboring the *nptII* and *ipt* genes were heterogeneous in their morphology and could be separated into the two groups differing in appearance. The first group plants were similar to control plants in their morphology but had a longer period of vegetative growth. The plants of the second group manifested increased tillering, low plant height, and the absence of fertile ears. Differences between transformants and control plants and between various transformants might be related to different expression of inserted *ipt* gene and correspondingly in different levels of cytokinins in plants. The plants of the first group were used for seed obtaining.

In experiments on root flooding, we also used plants of the first group. Two-week-long root flooding suppressed plants growth substantially and reduced productivity of both



Fig. 1 R₁ regenerated and control plants after 10 d flooding (t° = 32°C). 1. R₁ plants; 2. Control plants. For description see Table 1.

control and transgenic T₁ plants; however, these effects were less pronounced in transgenic plants. Thus, growth of control plants was suppressed by this stress by 14% stronger than growth of transgenic plants (Fig. 2). Especially strong difference was observed in the yield of transgenic and wild-type plants; it was equal to 36 and 2% of control plants, respectively (Fig. 3).

DISCUSSION

Our main strategy for obtaining plants tolerant to root flooding was creation of conditions for survival of the cells with improved energy and carbohydrate metabolism, which permitted them to survive oxygen deficiency. Therefore, sucrose was excluded from MS nutrient medium for selection of tolerant cells. Earlier we have demonstrated that in the absence exogenous carbohydrates sugarcane callus cells manifested high sensitivity to anoxia (Stepanova *et al.* 2002). Application of carbohydrate-free nutrient medium during anaerobic incubation should facilitate selection of the cells with a large store of endogenous carbohydrates or with their more efficient utilization in fermentation, and this

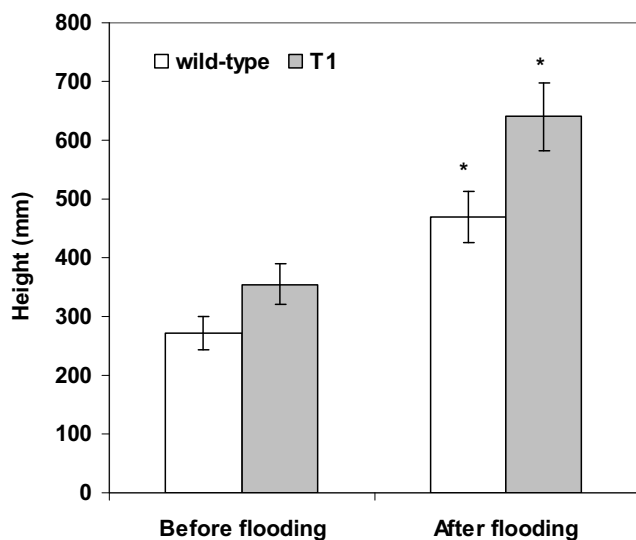


Fig. 2 Effect of root flooding on height of wild-type and transgenic wheat plants. Wild-type and T₁ generation of *ipt* transgenic wheat plants were planted in 9 l plastic vessels containing 4 kg of soil, 12 plants per vessel. Flooding was started during plant booting after termination of lower internode growth. Root flooding was achieved by watering with tap water so that a 5-cm water layer was above the soil surface. After 14 days flooding, the pots were drained and watered regularly like the control plants. Experiment on root flooding was repeated two times. Plant height before and after soil flooding was measured. Values are reported \pm SD ($n = 24$). Different letters on bars indicate significant differences between the wild-type and transgenic wheat plants values of the each conditions (before flooding or after flooding) at $P < 0.05$ (*) for T-test.

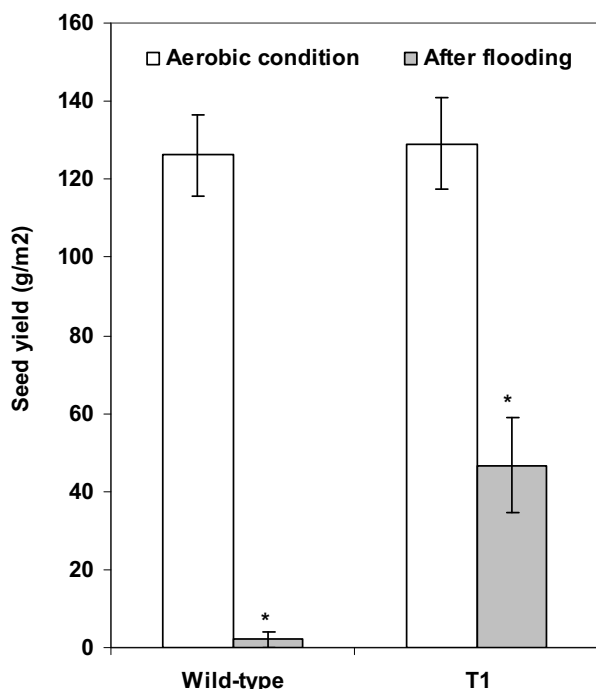


Fig. 3 Effect of root flooding on yield of wild-type and transgenic wheat plants. Yield for wild-type and T₁ generation of transgenic wheat plants was counted after 14-d flooding. Unflooded wild-type and T₁ generation were used as control plants (aerobic condition). Plant yield was determined after harvesting and weighing seeds. Values are reported \pm SD ($n = 24$). Different letters on bars indicate significant differences among the means at $P < 0.05$ (*) for LSD test.

permit these cells to maintain their energetic metabolism at the optimum level. In addition, more severe selective conditions in the absence of exogenous sugars permit much more rapid elimination of anaerobiosis-sensitive cells. Thus, *in vitro* selection of tolerant cells under conditions of anoxia

and in the absence of exogenous sources of carbohydrates could be realized due to the regulation of metabolism of endogenous carbohydrate. Tolerance of selected plants had a genetic nature, which was confirmed by inheritance of tolerance to soil flooding in the following seed generation. Thus, in these experiments, we demonstrated for the first time the inheritance of tolerance to soil flooding in plants obtained by *in vitro* cell selection (Table 1).

Along with *in vitro* selection of the cells tolerant to anaerobic stress that based on earlier demonstration of the key role of energy and carbohydrate metabolism in plant metabolic adaptation to hypoxia and anoxia, we also used a principally other approach for creating plants with improved tolerance to soil flooding, namely genetic engineering, which was earlier repeatedly used for production of plants tolerant to hypoxia and anoxia (Bucher *et al.* 1994; Tadege *et al.* 1998; Quimio *et al.* 2000; Zhang *et al.* 2000; Rahman *et al.* 2001; Ellis *et al.* 2003; Ismond *et al.* 2003). In most of these studies, to produce tolerant plants, they were transformed with genes of glycolysis and fermentation enzymes, pyruvate decarboxylase and alcohol dehydrogenase (Bucher *et al.* 1994; Tadege *et al.* 1998; Quimio *et al.* 2000; Rahman *et al.* 2001; Ellis *et al.* 2003; Ismond *et al.* 2003). However, because of complex mechanisms of the control of energetic metabolism, the results of these studies were ambiguous. Our experiments with transgenic plants were based on quite different idea. It is known that long-term soil flooding accelerates markedly leaf senescence and even death of such sensitive plants as wheat (Trought and Drew 1980). On the other hand, plant treatment with cytokinins results in a substantial delay of leaf senescence, in particular in the case of stress-induced senescence (Titov *et al.* 1986). Zhang *et al.* (2000) demonstrated that insertion of the *ipt* gene under the *SAG12* promoter improved tolerance to anaerobic stress of *Arabidopsis* plants. Taking into account the results of these studies, we attempted to retard wheat senescence induced by soil flooding by creating transgenic plants with activated cytokinin synthesis. We also took into consideration that, as distinct from arabidopsis, wheat is a monocotyledonous plant of a great economic importance resides and the literature concerning changes in the cytokinin content under oxygen deficit is rather contradictory (Bakhtenko *et al.* 2007).

We observed marked differences in the effects of 14-day-long soil flooding on wild-type and transgenic plants. The difference in plant yield was especially pronounced: the grain yield of flooded wild-type plants comprised only 2% of control plants, which did not experience flooding, whereas transgenic plant yield was 36% of control (Fig. 3). Keeping in mind that the average seed weight of wild-type and transgenic plants was approximately similar when plants were grown under conditions of normal soil aeration, it becomes evident that their distinct differences manifested under soil flooding indicate that the reason is a beneficial effect of stimulated cytokinin synthesis in transgenic plants.

These differences are also evident in the proportion of ears carrying seeds. Thus, in flooded wild-type plants, the proportion of ears with seeds comprised 33%, whereas 90% of transgenic ears had seeds.

In general, our experiments showed that plants obtained by *in vitro* cell selection and genetic engineering methods were more tolerant to root flooding, and this indicates that approaches used have a considerable promise.

REFERENCES

- Armstrong W, Brändle R, Jackson MB (1994) Mechanisms of flood tolerance in plants. *Acta Botanica Neerlandica* 43, 307-358
- Bakhtenko EYu, Skorobogatova IV, Karsunkina NP (2007) The role of hormonal balance during plant adaptation to flooding. *Proceedings of the Russian Academy of Sciences, Series Biology* 6, 682-690
- Bucher M, Brändle R, Kuhlemeier C (1994) Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase. *The EMBO Journal* 13, 2755-2763
- Collaku A, Harrison SA (2002) Losses in wheat due to waterlogging. *Crop Science* 42, 444-450
- Colmer TD (2003) Long-distant transport of gases in plants: a perspective on

- internal aeration and radial oxygen loss from roots. *Plant, Cell and Environment* **26**, 17-36
- Ellis MH, Millar AA, Llewellyn DJ, Peacock WJ, Dennis ES** (2003) Transgenic cotton (*Gossypium hirsutum*) over-expressing alcohol dehydrogenase shows increased ethanol fermentation but no increase in tolerance to oxygen deficiency. *Australian Journal of Plant Physiology* **27**, 1041-1050
- Ismail AM, Ella ES, Vergara GV, Mackill DJ** (2009) Mechanisms associated with tolerance to flooding during germination and early seedling growth in rice (*Oryza sativa* L.). *Annals of Botany* **103**, 197-209
- Ismond KP, Dolferus R, Pauw M, Dennis ES, Good AG** (2003) Enhanced low oxygen survival in *Arabidopsis thaliana* through increased metabolic flux in the fermentative pathway. *Plant Physiology* **132**, 1292-1302
- Jackson MB, Davies DD, Lambers H** (1991) *Plant Life under Oxygen Deprivation*, SPB Academic, The Hague, 326 pp
- Jackson MB, Black CR** (1993) *Interacting Stresses on Plants in a Changing Climate*, Springer-Verlag, NATO Asi Series, Berlin **16**, 771 pp
- Jackson MB, Armstrong W** (1999) Formation of aerenchyma and the processes of plant ventilation in relation to soil flooding and submergence. *Plant Biology* **1**, 274-287
- Jackson MB, Ishizawa K, Ita O** (2009) Evolution and mechanism of plant tolerance to flooding stress. *Annals of Botany* **103**, 137-142
- Kozłowski TT** (1984) Extent, causes and impact of flooding. In: Kozłowski TT (Ed) *Flooding and Plant Growth*, Academic Press, Orlando, Florida, USA, pp 1-5
- Loreti E, Yamaguchi J, Alpi A, Perata P** (2003) Sugar modulation of α -amylase genes under anoxia. *Annals of Botany* **91** (Special Issue), 143-148
- Magneschi L, Perata P** (2009) Rice germination and seedling growth in the absence of oxygen. *Annals of Botany* **103**, 181-186
- Maltby E** (1991) Wetlands – their status and role in the biosphere. In: Jackson MB, Davies DD, Lambers H (Ed) *Plant Life under Oxygen Deprivation. Ecology, Physiology and Biochemistry*, The Hague: SPB Academic, pp 3-21
- Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plantarum* **15**, 473-497
- Musgrave ME, Ding N** (1998) Evaluating wheat cultivars for waterlogging tolerance. *Crop Science* **38**, 90-97
- Quimio CA, Torrizo LB, Setter TL, Ellis M, Grover A, Abrigo EM, Oliva NP, Ella ES, Carpena AL, Ito O** (2000) Enhancement of submergence tolerance in transgenic rice overproducing pyruvate decarboxylase. *Journal of Plant Physiology* **156**, 516-521
- Rahman M, Glover A, Peacock WJ, Dennis ES, Ellis MH** (2001) Effects of manipulation of pyruvate decarboxylase and alcohol dehydrogenase levels on the submergence tolerance of rice. *Australian Journal of Plant Physiology* **28**, 1231-1241
- Romanov GA** (2009) How do cytokinins affect the cell? *Russian Journal of Plant Physiology* **56**, 286-290
- Sachs MM, Vartapetian BB** (2007) Plant anaerobic stress I. Metabolic adaptation to oxygen deficiency. *Plant Stress* **1**, 123-135
- Setter TL, Waters I** (2003) Review of prospects for germplasm improvement for waterlogging tolerance in wheat, barley and oats. *Plant and Soil* **253**, 1-34
- Setter TL, Waters I, Sharma SK, Singh KN** (2009) Review of wheat improvement for waterlogging tolerance in Australia and India: the importance of anaerobiosis and element toxicities associated with different soils. *Annals of Botany* **103**, 221-236
- Stepanova AY, Polyakova LI, Dolgikh YI, Vartapetian BB** (2002) The response of sugarcane (*Saccharum officinarum*) cultured cells to anoxia and the selection of a tolerant cell line. *Russian Journal of Plant Physiology* **49**, 406-412
- Stepanova AY, Tereshonok DV, Ossipova ES, Gladkov EA, Dolgikh Yul** (2006) Production of wheat (*Triticum aestivum* L.) transgenic plants by *Agrobacterium*-mediated transformation. *Biotechnology (Russia)* **2**, 20-27
- Tadege M, Brändle R, Kuhlemeier C** (1998) Anoxia tolerance in tobacco roots: effect of overexpression of pyruvate decarboxylase. *The Plant Journal* **14**, 327-335
- Titov AF, Drozdov SN, Kritenko SP, Talanova VV, Sherudilo EG** (1986) Cytokinin influence on cold resistance of active developing plants. *Physiology and Biochemistry of Crop Plants (Russia)* **18**, 64-69
- Trought MCT, Drew MC** (1980) The development of waterlogging damage in wheat seedlings (*Triticum aestivum* L.) I. Shoot and root growth in relation to changes in the concentrations of dissolved gases and solutes in the soil solution. *Plant and Soil* **70**, 110-112
- Vartapetian BB** (1978) Introduction: life without oxygen. In: Hook DD, Crawford RMM (Ed) *Plant Life in Anaerobic Environments*, Ann Arbor Science, Ann Arbor, Michigan, pp 1-12
- Vartapetian BB, Jackson MB** (1997) Plant adaptation to anaerobic stress. *Annals of Botany* **79** (Special Issue), 3-20
- Vartapetian BB, Sachs M, Fagerstedt KV** (2008) Plant anaerobic stress. II. Strategy of avoidance of anaerobiosis and other aspects of plant life under hypoxia and anoxia. *Plant Stress* **2**, 1-19
- Visser EJW, Voeselek LACJ, Vartapetian BB, Jackson MB** (2003) Flooding and plant growth. *Annals of Botany* **91** (Special Issue), 107-109
- Zhang J, Van Toai T, Huynh L, Preiszner J** (2000) Development of flooding-tolerant *Arabidopsis thaliana* by autoregulated cytokinin production. *Molecular Breeding* **6**, 135-144