Efficient callus formation and plant regeneration of goosegrass [Eleusine indica (L.) Gaertn.]

Abstract Efficient methods in totipotent callus formation, cell suspension culture establishment and whole-plant regeneration have been developed for the goosegrass [Eleusine indica (L.) Gaertn.] and its dinitroaniline-resistant biotypes. The optimum medium for inducing morphogenic calli consisted of N6 basal salts and B5 vitamins supplemented with 1–2 mg l–1 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg l –1 glycine, 100 mg l –1 asparagine, 100 mg l–1 casein hydrolysate, 30 g l–1 sucrose and 0.6% agar, pH 5.7. The presence of organogenic and embryogenic structures in these calli was histologically documented. Cell suspension cultures derived from young calli were established in a liquid medium with the same composition. Morphogenic structures of direct shoots and somatic embryos were grown into root- ed plantlets on medium containing MS basal salts, B5 vitamins, 1 mg l–1 kinetin (Kn) and 0.1 mg l–1 indole-3-acetic acid (IAA), 3% sucrose, 0.6% agar, pH 5.7. Calli derived from the R-biotype of E. indica possessed a high resistance to trifluralin (dinitroaniline herbicide) and cross-resistance to a structurally non-related herbicide, amiprophosmethyl (phosphorothioamidate herbicide), as did the original resistant plants. Embryogenic cell suspension culture was a better source of E. indica protoplasts than callus or mesophyll tissue. The enzyme solution containing 1.5% cellulase Onozuka R-10, 0.5% driselase, 1% pectolyase Y-23, 0.5% hemicellulase and N6 mineral salts with an additional 0.2 M KCl and 0.1 M CaCl2 (pH 5.4–5.5) was used for protoplast isolation. The purified protoplasts were cultivated in KM8p liquid medium supplemented with 2 mg l–1 2,4-D and 0.2 mg l–1 Kn.

Keywords Eleusine indica · Somatic embryogenesis · Organogenesis · Dinitroaniline herbicides · Cross resistance · Protoplasts

Abbreviations Asp: Asparagine · BA: 6-Benzylaminopurine · 2,4-D: 2,4-Dichlorophenoxyacetic acid · DNH: Dinitroaniline herbicides · Gly: Glycine · IAA: Indole-3-acetic acid · Kn: Kinetin

Introduction

Goosegrass [Eleusine indica (L.) Gaertn.] is one of the most widespread weeds occurring in croplands. To date, the dinitroaniline herbicides (for example, trifluralin, oryzalin, pendimethalin, ethalfluralin) have been widely used to control goosegrass in arable crops (Smeda and Vaughn 1994; Vaughn 2000). The dinitroaniline herbicides target tubulin, which is the principle constituent protein of microtubules (MTs) (Vaughn 2000). The general mechanism of DNH is to alter the ability of tubulin to polymerise into MTs, and the inhibition of this polymerisation process results in the eventual loss of all MTs (Smeda and Vaughn 1994; Vaughn 2000). MTs are crucial for such cellular processes as cell-shape establishment, chromosome movement during mitosis and cell-plate formation (Smeda and Vaughn 1994; Vaughn 2000).

Repeated application of DNH on field-grown crops is considered to be the causal factor for the appearance of resistant (R) biotypes of E. indica from susceptible (S) ones (Mudge et al. 1984). The highly resistant biotype has a 1,000–10,000-fold higher resistance to trifluralin than the S-biotype and also exhibits cross-resistance to another DNH as well as to the phosphorothioamide herbicide amiprophosmethyl (APM) (Smeda and Vaughn 1994). Genetic segregation analyses on F1 hybrids and F2 and F3 generations indicated that the DNH-resistance phenotype is inherited as a single, recessive nuclear gene (Zeng and Baird 1997). Moreover, both Cronin et al.
(1993) and Yamamoto et al. (1998) proved independently that resistance to dinitroanilines in the R-biotype of *E. indica* is related with α-tubulin (*TUA1*) missense mutation, which converts Thr-239 to Ile in the R-biotype.

Since DNH are still commonly used in agriculture, the production of dinitroaniline-resistant crops may be of practical interest (Baird et al. 2000). One of the ways to produce such plants is to transfer the trait of DNH resistance from corresponding sources (mutants) to recipients using cellular (Yemets et al. 1997; Yemets and Blume 1999) or genetic engineering (Antony et al. 1998) techniques. To date, it has been impossible to obtain genetically modified regenerated monocot plants with resistance to DNH due to peculiarities of the auto-regulatory mechanisms of α- and β-tubulin co-expression, which is probably suppressed by transformation with exogenous tubulin genes, especially under powerful promoters (Yemets and Blume 1999). Taking into account the importance of cellular engineering methods for the transfer of the desirable traits, we have established in vitro culture system for the DNH-resistant biotypes of *E. indica*. Such cultures can be suitable sources for the transfer of DNH resistance into different cereals by, for example, somatic hybridisation or micro-nucleation.

In vitro cultures of such biotypes would also be useful material for cell biology studies. For example, the embryogenic goosegrass model developed here could be used to investigate: (1) the tissue specificity of tubulin gene expression, especially of mutant α-tubulin, during differentiation and mechanisms involved in the regulation of tissue-specific expression of mutant tubulin; (2) the possibility to produce mini-chromosomes (via micro-protoplasting) for further creation of artificial chromosomes with tubulin genes; (3) peculiarities of microtubular organisation in the processes of differentiation and embryogenesis (using specific monoclonal antibodies produced against altered tubulin).

As new crop varieties of this weedy species have been recently developed in the Ukraine, these newly developed protocols for cell and tissue culture establishment, as well as for protoplast isolation, may also form the practical basis for further in vitro manipulations with these new *E. indica* varieties. These procedures may be first step in the biotechnological development of herbicide-resistant goosegrass crops as well as an approach for improving such closely related agricultural species as finger millet, *E. coracana*.

In this paper we report the development of methods for inducing totipotent calli in vitro, resulting in our obtaining cell suspension cultures and regenerating R- and S-biotype plants. We also describe the peculiarities of the regeneration processes of *E. indica* via somatic embryogenesis and organogenesis under in vitro conditions by presenting data from a histological analysis. Our results revealed the preservation of resistance to trifluralin and cross-resistance to APM in established totipotent calli of the *E. indica* R-biotype. We also present our elaborated method for goosegrass protoplast isolation.

### Materials and methods

#### Plant material

*Eleusine indica* seeds of DNH-resistant biotype RGG and susceptible biotype SGG were obtained from the American Cyanamid Co. (New Jersey), and DNH-resistant biotypes MSC-R and MGA-R and susceptible biotypes ASC-S and GTN-S were kindly provided by Prof. W.V. Baird (Clemson University, S.C.). Coleoptiles, mesocotyles and young leaves from seedlings of both R- and S-biotypes were used as initial explants for callus induction.

#### Herbicides

Trifluralin [2,6-dinitro-4-(trifluoromethyl)benzenamine] was obtained from Dr. L. Guse (DowElanco, Greenfield, USA). AMP, also known as Bay NTN 6867, was obtained from Dr. J.R. Bloomberg (Agriculture Division, Miles, Kansas City, Kan.). The preparation of the herbicide stock solutions and their addition into media were carried out as described by Yemets et al. (2000).

#### Seed sterilisation and germination

Seeds were surface-sterilised for 7 min in 70% (v/v) ethanol, followed by 10 min in hypochlorite, and then washed twice for 20 min each time in sterilised distilled water and once for 10 min in media-A (a combination of different mineral salts; W.V. Baird, personal communication). The seeds were then germinated under sterile conditions on filter papers moistened with medium-A in petri dishes. The dishes were incubated in darkness for the initial 12 h at 36–37°C and then at 25°C for 3–5 days.

#### Basal medium and incubation conditions

The basal medium used in this investigation consisted of N6 basal salts (Chu et al. 1975) and B5 vitamins (Gambaro et al. 1968) supplemented with 30 g l–1 sucrose. Except for the cell suspension medium, all media were solidified with 6 g l–1 agar. The pH of all media was adjusted to 5.7 before autoclaving. All cultures were inoculated in controlled environment chambers at 25±1°C.

#### Callus culture

The following phytohormones and medium supplements were tested for callus induction: (1) 2,4-D at concentrations of 1–6 mg l–1 and 30 g l–1 sucrose; (2) 1 mg l–1 2,4-D, 0–0.1 mg l–1 Kn and different organic supplements (Table 1). After induction, the calli were maintained on the same media or on media with reduced 2,4-D (from 2.5 mg l–1 to 2 mg l–1, or from 2 mg l–1 to 1–1.5 mg l–1), depending upon the biotype. Calli were incubated in darkness.

#### Cell suspension cultures

To establish suspension cell cultures, we transferred callus, at early stages of formation, to liquid basal medium supplemented with 1–2 mg l–1 2,4-D, 2 mg l–1 Gly, 100 mg l–1 Asp and 100 mg l–1 casein hydrolysate. Suspension cultures were incubated in 250–ml Erlenmeyer flasks containing 80 ml of liquid medium on a shaker at 180 rev min–1.

#### Development of morphogenic structures

Calli with morphogenic structures at early developmental stages were transferred either directly to development medium, or first to...