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Callus induction and plant regeneration from different explant types of *Miscanthus* × *ogiformis* Honda 'Giganteus'

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Abstract

Different explants of *Miscanthus* × *ogiformis* Honda 'Giganteus' were tested in order to develop an efficient tissue culture system. Shoot apices, leaf and root sections from *in vitro*-propagated plants, and leaf and immature inflorescence sections from 6-month-old greenhouse-grown plants were used. The explants were cultured on Murashige and Skoog medium supplemented with 4.5, 13.6, 22.6 or 31.7 μM 2,4-dichlorophenoxyacetic acid. Three types of callus were formed but only one was embryogenic and regenerated plants. Callus induction and formation of embryogenic callus depended on the type and developmental stage of the explants. Shoot apices formed the highest percentage of embryogenic callus. There was a difference in the formation of embryogenic callus between leaf explants from *in vitro*-propagated shoots and greenhouse-grown plants. The best results were obtained from newly formed leaves of *in vitro*-propagated shoots and older leaves of greenhouse-grown plants. Immature inflorescences smaller than 2.5 cm produced a higher percentage of embryogenic callus than larger more mature inflorescences. Embryogenic callus derived from immature inflorescences had the highest regeneration capacity. Differences in 2,4-dichlorophenoxyacetic acid concentrations had no significant effect on callus induction, embryogenic callus formation and plant regeneration.

Abbreviations: MS – Murashige & Skoog; 2,4-D – 2,4-dichlorophenoxyacetic acid; BA – benzyladenine; NAA – 1-naphthaleneacetic acid; PPFD – photosynthetic photon flux density

Introduction

Miscanthus is a temperate perennial cross-pollinating grass used commercially as an ornamental plant. *Miscanthus* × *ogiformis* Honda 'Giganteus' is a triploid hybrid between the tetraploid *M. sacchariflorus* and the diploid *M. sinensis* (Linde-Laursen, 1993). The large biomass production, and the low input of fertilizer needed (Schwarz *et al.*, 1994) makes the Giganteus clone an interesting non-food crop. The clone can be vegetatively propagated by rhizome division (Nielsen, 1987) or by *in vitro* propagation by axillary shoots (Nielsen *et al.*, 1993, 1995). Development of an efficient tissue culture system for somatic embryogenesis could provide an alternative propagation method and be useful for breeding purposes.

Callus culture systems regenerating plantlets through somatic embryogenesis have been developed in many species of Poaceae (Vasil, 1987; Lörz *et al.*, 1988). Only young immature tissues can be used as explants for callus induction because the tissue looses its competence for callus induction with age. Hence, embryogenic calli have successfully been induced on immature embryos, immature inflorescences, young leaves, shoot apices and root tips (Vasil, 1987; Lörz *et al.*, 1988). Considerable differences have been observed when different explant types of the same genotype have been compared for the capacity to produce embryogenic callus and to regenerate plants. In wheat, inflorescences produced embryogenic callus which regenerated a higher number of plants than callus produced on leaf explants (Tabaeizadeh *et al.*,

1990). Differences in the capacity to form embryogenic callus were also found between leaves and immature embryos in barley (Ruíz *et al.*, 1992) and between immature embryos and inflorescences in wheat (Maddock *et al.*, 1983).

The same levels of 2,4-D can have different effects on callus induction and callus quality on explants from different genotypes (Carman, 1990). In grasses, the optimal 2,4-D concentration for embryogenic callus formation in leaves (Wernicke & Milkovits, 1984; Barcelo *et al.*, 1992) and in immature inflorescences (Brettell *et al.*, 1980; Creemers-Molenaar *et al.*, 1988) was found to be dependent on the genotype and on the developmental stage of the explants. Callus induction and plant regeneration from different explants of greenhouse-grown *Miscanthus* 'Giganteus' plants have previously been investigated using callus induction media with different combinations of cytokinin and auxin (Petersen & Holme, 1992; Lewandowski & Kahnt, 1993).

In order to develop an efficient system for somatic embryogenesis in *M.* × *ogiformis* Honda 'Giganteus', the callus induction capacity of different types and developmental stages of explants was investigated. The embryogenic competence of the callus types and the regeneration capacity of the embryogenic calli were evaluated. Furthermore, the effects of different concentrations of 2,4-D on callus induction and quality were investigated. In the present study, the addition of cytokinin was avoided since cytokinins often induced shoot-forming callus, making long-term embryogenic callus harder to select and maintain.

Materials and methods

Plant material

Plant material of *Miscanthus* × *ogiformis* Honda 'Giganteus' was obtained from *in vitro*- and greenhouse-grown plants. The following explant types were used: shoot apices, leaves and roots from *in vitro*-grown plants and leaves and immature inflorescences from greenhouse-grown plants. *In vitro* shoots were propagated according to Nielsen *et al.* (1993) with 22.2 μM BA and 1.3 μM NAA. Roots were induced according to Nielsen *et al.* (1993).

Greenhouse-grown plants originated from *in vitro*-propagated plantlets. In January the plantlets were potted and acclimatized in a controlled environment room under a polyethylene tent at a basal temperature of 23

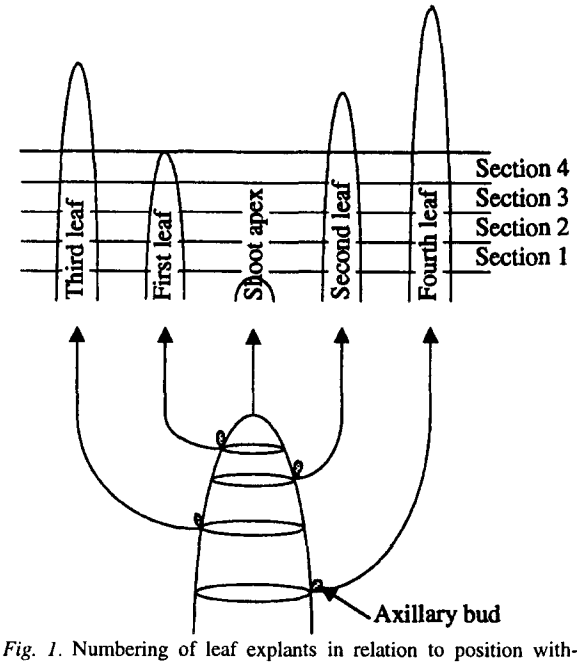

Fig. 1. Numbering of leaf explants in relation to position within shoots from *in vitro*-grown or greenhouse-grown plants of *Miscanthus* × *ogiformis* Honda 'Giganteus'. (Modified drawing after Guiderdoni & Demarly, 1988).

± 0.5 °C, a photoperiod of 16-h and a PPFD of 35 μmol m^{-2} s^{-1} provided by fluorescent tubes (Pope type FSD 58 W/33). After 14 days of acclimatization, plants were transferred to a greenhouse and grown at a minimum temperature of 28 °C and natural light conditions until the appearance of immature inflorescences in July/August.

Explant types

Greenhouse-grown material was surface sterilized in 0.5% sodium hypochlorite for 2 min and rinsed three times with sterile water. All explant types, except immature inflorescences shorter than 2 mm and shoot apices, were cut into 2 mm sections and placed on the callus induction medium. Shoot apices (1 mm) were dissected from *in vitro*-propagated shoots. Leaf explants were dissected from the four youngest leaves of *in vitro*-propagated shoots 4 weeks after subculture and of greenhouse-grown plants having 8–10 visible leaves. Leaf explants were placed in positional order in Petri dishes to evaluate the effects of the developmental stage (Fig. 1). Root explants from the five most apical 2 mm sections of roots were placed on the callus induction medium in sequential order to evaluate the effects of developmental stage. Only immature inflorescences

between 0.05 and 65 mm in length were used, ensuring that no meiosis had occurred. All explants obtained from one inflorescence were placed in the same Petri dish.

Callus induction and culture

The callus induction medium was MS basal medium (Murashige & Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 2 g l^{-1} gelrite, 750 mg l^{-1} $MgCl_2 \cdot 6H_2O$ and varying 2,4-D concentrations: 4.5, 13.6, 22.6 or 31.7 μM. In all media used in this study, the pH was adjusted to 5.5 prior to autoclaving. Explants were incubated in darkness at 27 °C in 9 cm glass Petri dishes sealed with cling film and subcultured every second week. Eight weeks after culture initiation the number of explants with callus was recorded and the calli were classified into three different types with respect to appearance. Concerning immature inflorescences, the total number of calli induced and the callus types were recorded for each inflorescence. The callus induction percentage was calculated as: Callus induction percentage = (number of explants with callus / total number of explants) × 100. The percentage of each callus type was calculated as: Callus type percentage = (number of specific callus type / total number of calli) × 100.

Plant regeneration

Eight to twelve weeks after explant excision, plant regeneration, was initiated on MS basal medium with 30 g l^{-1} sucrose, 750 mg l^{-1} $MgCl_2 \cdot 6H_2O$, 2 g l^{-1} gelrite and 8.8 μM BA. Regeneration took place at 27 °C under a PPFD of 6.7 μmol m^{-2} s^{-1} and callus was subcultured after one month. After two months on regeneration medium, the number of plants regenerated per callus (0.5 cm in diameter) was recorded.

Statistics

For analysis of variance, the GENMOD Procedure of SAS/STAT Software (SAS, 1993) was used. All data were analyzed using the gamma distribution and the link function log was used to transform the data.

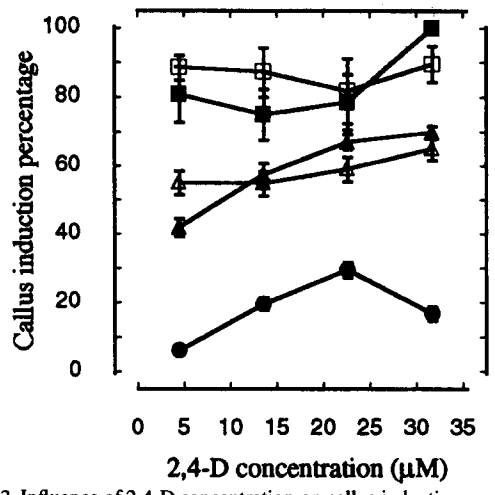

Fig. 2. Influence of 2,4-D concentration on callus induction percentage in explants from immature inflorescences (□) [50], shoot apices of *in vitro*-grown plants (■) [25], roots of *in vitro*-grown plants (△) [180], leaves of greenhouse-grown shoots (▲) [150] and leaves of *in vitro*-grown shoots (●) [350]. The numbers in brackets are the minimum number of explants used at each 2,4-D concentration. Bars represent ± standard errors.

Results

Callus induction on different explant types

The highest callus induction percentages were obtained on explants from immature inflorescences or shoot apices (Fig. 2). Root explants from *in vitro* plants and leaf explants from shoots of greenhouse-grown plants also showed relatively high callus induction. Less than 30% of the leaf explants from *in vitro*-grown shoots produced callus.

Addition of 2,4-D to the medium was required for induction of callus. The concentrations of 2,4-D used, however, significantly affected callus induction only on leaf explants from greenhouse-grown ($p<0.0001$) or *in vitro*-grown shoots ($p<0.0001$) (Fig. 2). Increasing 2,4-D concentration from 4.5 to 31.7 μM, increased the callus induction percentage from 41.9 to 69.9% on explants from greenhouse-grown leaves. The maximum callus induction on leaf explants from *in vitro*-propagated shoots was 29.5% at 22.6 μM 2,4-D.

The formation of callus on leaf explants from 4-week-old *in vitro*-grown shoots was significantly affected by leaf number ($p<0.0001$) and leaf section ($p<0.0001$) (Fig. 1 and 3a) and was higher on explants from young leaves and proximal leaf sections than on explants from older leaves and distal leaf sections.

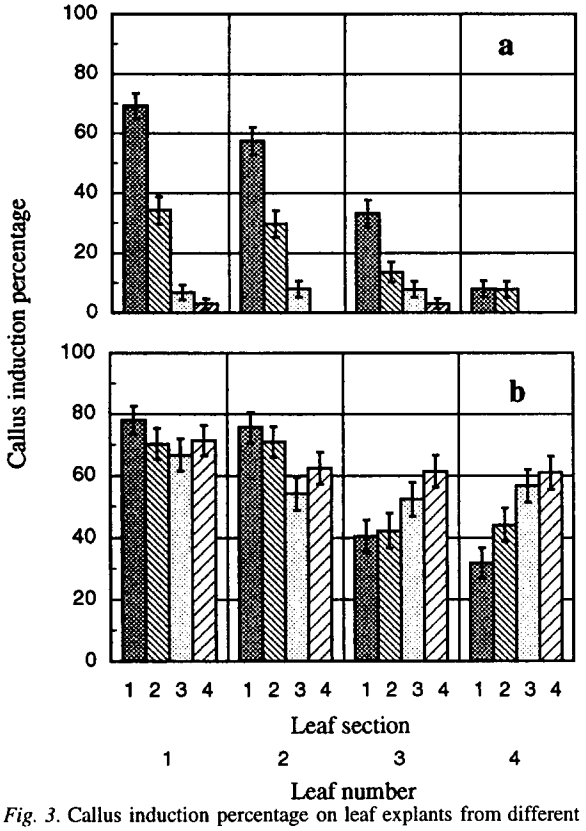

Fig. 3. Callus induction percentage on leaf explants from different positions within *(a) in vitro*-grown shoots or *(b)* greenhouse-grown shoots. The minimum numbers of explants excised for each explant position were 98 and 34 respectively. For explanation see Fig. 1. Bars represent ± standard errors.

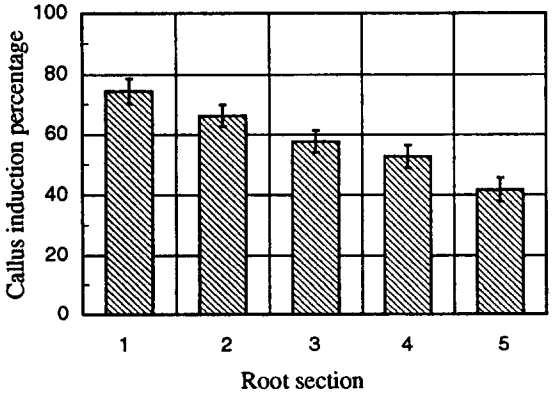

Fig. 4. Callus induction percentage on explants from *in vitro*-grown roots. Root section 1 is the 2 mm section comprising the root meristem. Sections 2, 3, 4 and 5 are the 2 mm sections successively following the explant with the meristem. Values are means of at least 160 explants per section. Bars represent ± standard errors.

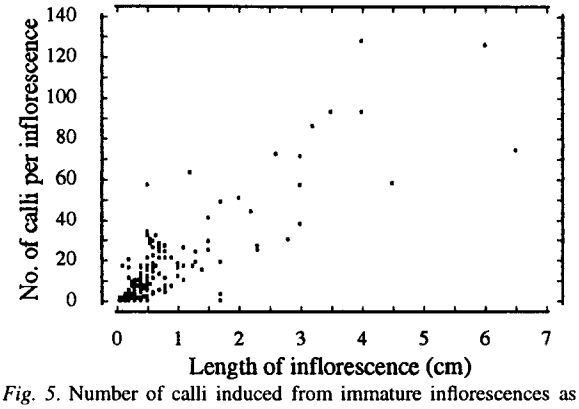

Fig. 5. Number of calli induced from immature inflorescences as affected by the length. Each point represents the total number of calli induced from each inflorescence. The plot represents the results for 204 inflorescences.

Interaction between leaf number and leaf section was also found ($p<0.0001$).

Formation of callus on leaf explants from greenhouse-grown plants also decreased with leaf number ($p<0.0001$, Fig. 3b) but did not show the same steep decrease as *in vitro*-grown leaves. A significant interaction between leaf number and leaf section was found ($p<0.0229$). The callus induction percentage decreased from proximal to distal sections on the two youngest leaves but increased on the two older leaves.

Callus induction on root explants was significantly affected by the position of the explant ($p<0.0002$, Fig. 4). The explant comprising the root meristem showed the highest callus induction percentage. The formation of callus decreased with increasing distance from the apex.

For immature inflorescences, the number of calli induced increased significantly ($p<0.0001$, Fig. 5) with length. The number of calli originating from each 2-mm section of an inflorescence was not determined. Direct comparison to other explant types is therefore not possible.

Callus types from different explants

Callus appeared two weeks after culture initiation and during the following weeks different callus types were formed. Eight weeks after culture initiation three types of callus could be identified resembling descriptions of callus types appearing in other grass tissue culture systems (reviewed by Morrish *et al.*, 1987): K1, a nodular, semisoft callus sometimes with anthocyanin colored spots which formed roots upon transfer to lower 2,4-D concentrations; K2, a compact, opaque

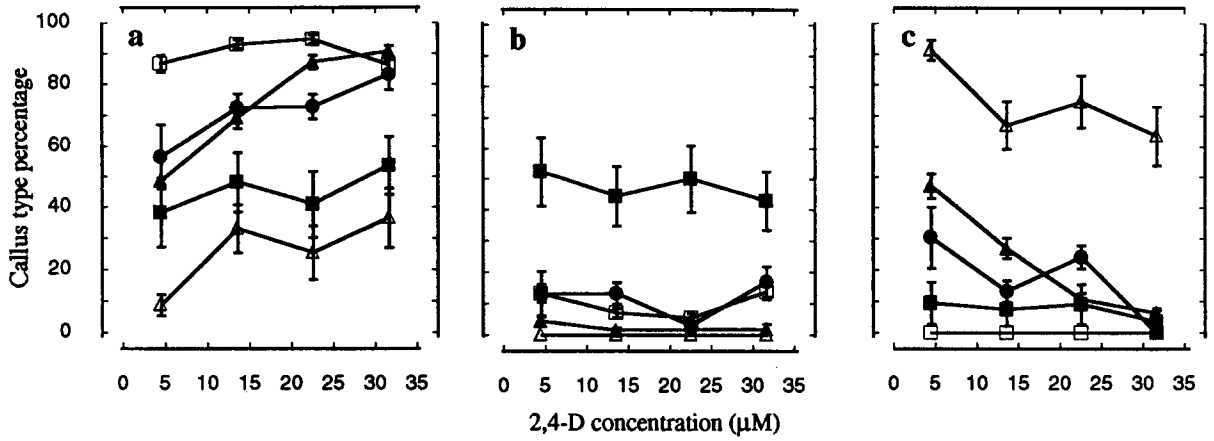

Fig. 6. Callus type percentages on different explant types as affected by the 2,4-D concentration. *(a)* K1 callus, *(b)* K2 callus and *(c)* K3 callus. Immature inflorescences (□) [2390], shoot apices (■) [98], roots (△) [497], greenhouse leaves (▲) [809] and *in vitro* leaves (●) [312]. The numbers in brackets are the total number of calli induced from each explant type. Bars represent ± standard errors.

white callus resembling the compact non-differentiated embryogenic callus described in other grasses and assigned Type I in maize (Ho & Vasil, 1983; Vasil & Vasil, 1986); K3, a soft, friable callus, translucent to milky in appearance resembling the nonmorphogenic and nonembryogenic friable callus described in other grasses (Morrish *et al.*, 1987). Immature inflorescences also developed a low percentage of a shoot-forming callus type. This callus type was not included in this study.

Calli from immature inflorescences formed the highest percentage of K1 callus (Fig. 6a). The percentage of K2 callus formed on this explant type was relatively low (Fig. 6b) and no K3 callus was formed. Shoot apices formed the highest percentage of K2 callus (Fig. 6b). Almost equal percentages of K2 and K1 callus and only a small percentage of K3 callus were formed from shoot apices. Roots formed the highest percentage of K3 callus, a low percentage of K1 callus and no K2 callus. The concentration of 2,4-D did not significantly affect the callus type percentages from immature inflorescences, shoot apices and roots (Fig. 6a, b, c).

Leaf explants from greenhouse-grown plants and *in vitro* shoots formed relatively high percentages of K1 callus. The K2 callus percentage was low but higher on leaf explants from *in vitro*-propagated shoots than from greenhouse-grown shoots. The percentage of K1 callus on leaves from greenhouse-grown shoots increased significantly with increasing 2,4-D concentration ($p<0.0001$, Fig. 6a). The same tendency was found for *in vitro*-grown shoots. The percentage of K3 callus decreased with increasing 2,4-D concentration on leaves from *in vitro*- ($p<0.0001$) or greenhouse-grown shoots ($p<0.0001$) (Fig. 6c). The percentage of K2 callus on leaves from greenhouse-grown shoots showed a significant but small decrease with increasing concentration of 2,4-D ($p<0.0001$) from 4.5% at 4.5 μM to 2.0% at 31.7 μM 2,4-D.

Embryogenic callus formation on explants of different developmental stages

The compact white callus type K2 was the only embryogenic callus type induced on explants under the conditions used in this study. Significant effects of leaf number ($p<0.0419$) and leaf section ($p<0.0269$) were found on the formation of embryogenic callus on leaf explants from *in vitro*-propagated shoots. Embryogenic callus was only formed on the three most proximal sections of the youngest leaf, the two most proximal sections of the second leaf and the most proximal section of the third leaf (Fig. 7a).

The formation of K2 callus on leaf explants of greenhouse-grown shoots showed a different pattern (Fig. 7b). Sections of leaf number 4 formed the highest percentage of K2 callus.

The percentage of K2 callus decreased with increasing length of the immature inflorescence but no statistically significant effect was found. The formation of K2 callus on inflorescences shorter than 2.5 cm was 13.1% on average, ranging from 0 to 62% whereas the formation of K2 callus on inflorescences longer than 2.5 cm was 5% on average, ranging from 0 to 14%.

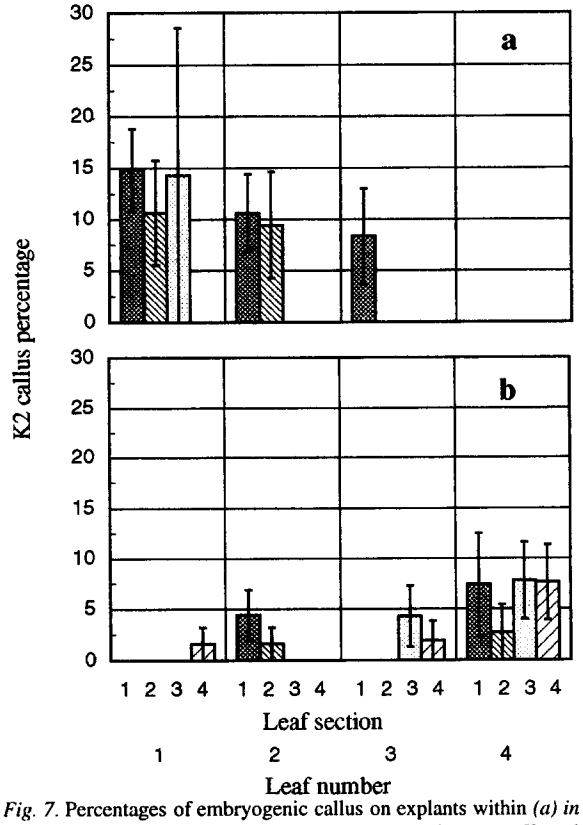

Fig. 7. Percentages of embryogenic callus on explants within *(a) in vitro*-propagated shoots or *(b)* greenhouse-grown shoots as affected by leaf number and leaf section. For explanation see Fig. 1. Bars represent ± standard errors.

Table 1. Frequency of plant regeneration from embryogenic calli induced on different explant types as affected by 2,4-D concentration. Plant regeneration was estimated as the number of plants regenerated per 0.5 cm diameter callus (average fresh weight 60 mg). The numbers in parentheses are the number of calli transferred to the regeneration medium.

2,4-D conc. in induction medium (μM)	*In vitro* leaves and Shoot apices	Greeenhouse leaves	Immature inflorescences
4.5	0.26 (19)	0.04 (26)	0.47 (19)
13.6	0.23 (13)	0.13 (8)	0.46 (11)
22.6	0.19 (37)	0.00 (7)	0.41 (22)
31.7	0.17 (6)	0.43 (7)	0.67 (12)

Plant regeneration

Only minor differences were found between the number of plants regenerated from embryogenic calli induced on shoot apices and on leaves of *in vitro*-propagated shoots. Therefore, an average of the plant regeneration from these two explant types is shown (Table 1). All plants regenerated in this investigation were green. No clear effect of the 2,4-D concentration in the callus induction medium was observed on the number of plants regenerated per callus. In general, embryogenic callus induced from immature inflorescences regenerated the highest number of plants among the explants tested. Furthermore, the embryogenic callus from inflorescences was less compact and somatic embryos were easier to distinguish (Fig. 8a). Plants regenerated from all explant types were successfully acclimatized and transferred to soil (Fig. 8b, c).

Discussion

Consistent with reports for other grasses (Morrish *et al.*, 1987), callus induction and embryogenic callus formation in *Miscanthus* × *ogiformis* Honda 'Giganteus' were dependent on explant type and the developmental stage of the explant. In agreement with results obtained from other grasses (Wernicke & Milkovits 1986; Bhaskaran & Smith, 1988), the most immature explant, the shoot apex, showed the highest callus induction and the highest embryogenic callus percentage of all explant types tested.

The high callus induction percentage on the most basal 2 mm section of the youngest leaf from 4-week-old *in vitro* shoots, with a steep decrease towards older leaves and more distal sections, and the development of embryogenic callus on only the very basal explants of the youngest leaves are consistent with results obtained by Guiderdoni & Demarly (1988) on young *in vitro*-grown shoots of sugarcane, and on 10-day-old seedlings of sorghum (Wernicke & Brettell, 1980) and rice (Wernicke *et al.*, 1981).

The gradual decrease in callus induction with age of leaves from 6-month-old greenhouse-grown *Miscanthus* plants and the small difference in callus induction percentage among the basal sections from the two youngest leaves indicate that young leaves from adult greenhouse-grown plants have an extended basal meristematic region in comparison with leaves of 4-week-old *in vitro*-propagated shoots. In support of this, Wernicke & Milkovits (1984) have shown that the extension of the meristematic region during the development of the second foliage leaf of 6- to 18-day-old wheat seedlings resulted in an extended region of leaf sections forming callus. The increase in callus induction percentage among the basal sections of the third and the fourth leaf was found to correlate with the for-

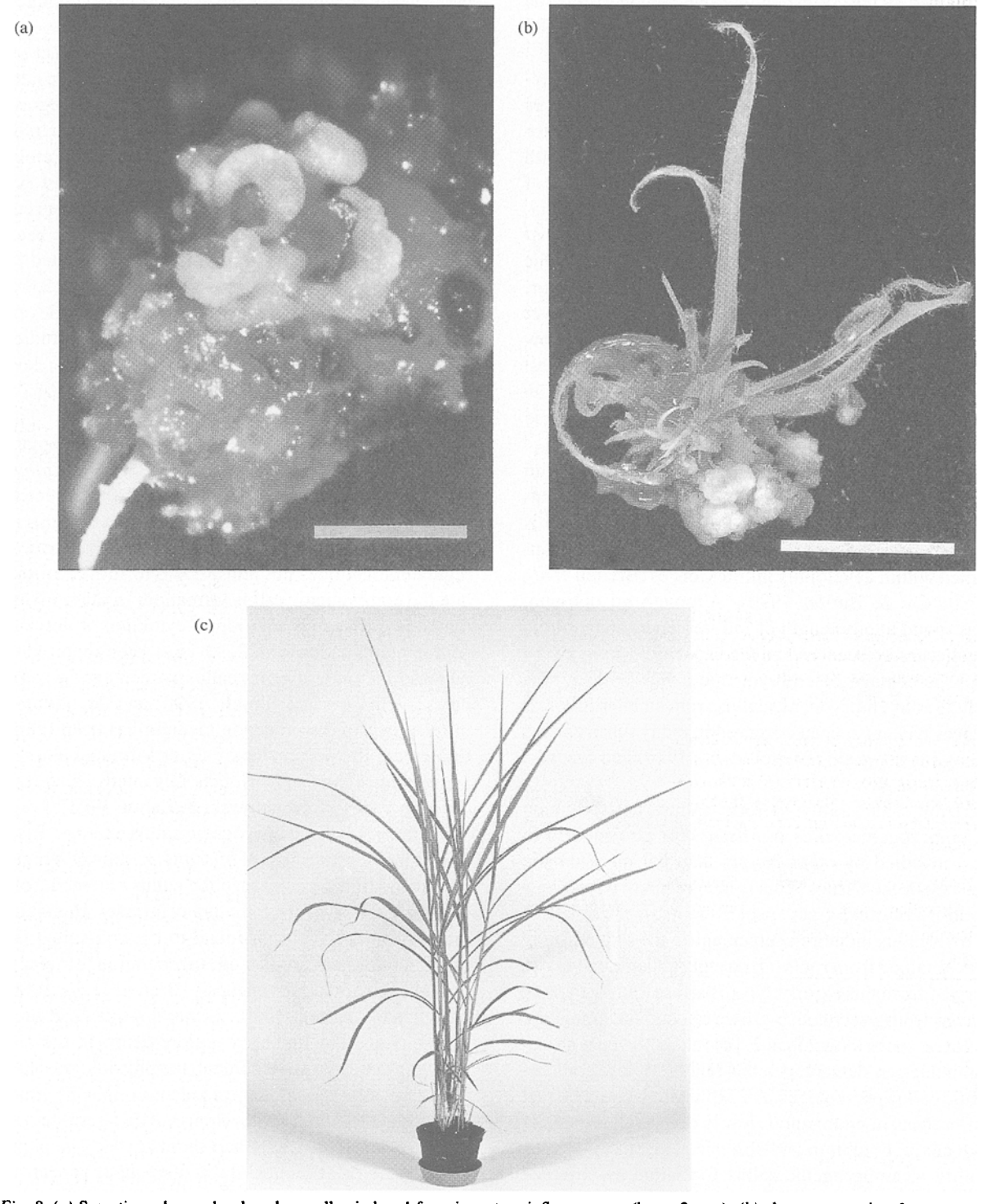

Fig. 8. (a) Somatic embryos developed on callus induced from immature inflorescences (bar = 2 mm), *(b)* plant regenerating from compact embryogenic K2 callus (bar = 10 mm) and *(c)* regenerated plant transferred to soil.

mation and growth of the leaf sheath, and might be explained by the corresponding switch in meristematic activity from the leaf blade meristem to the leaf sheath meristem (Barcelo *et al.*, 1991).

The development of embryogenic callus on all sections from the most mature of the four youngest leaves of greenhouse-grown shoots resembles the results for adult sugarcane plants where the fourth and fifth leaves were found to produce the highest percentage of embryogenic callus (Ho & Vasil, 1983). An explanation for the maturity related embryogenic callus formation could be that cell proliferation and embryogenic callus formation often are associated with the cells surrounding the vascular system (Lu & Vasil, 1981; Ho & Vasil, 1983; Guiderdoni & Demarly, 1988). The low embryogenic callus induction from the three youngest leaves of sugarcane was found to correlate with an incomplete differentiation of the xylem and phloem in these leaves (Ho & Vasil, 1983).

The increase in callus induction percentage with increasing length of immature inflorescences is consistent with results obtained for other grasses and is explained by the many active meristematic cells contained within developing inflorescences (Brettell *et al.*, 1980; Cai & Butler, 1990). Although no optimum was found at any length of inflorescence, some of the smaller inflorescences had the capacity to form a very high percentage of embryogenic callus. Higher percentages of embryogenic callus from smaller inflorescences have also been observed in many other grasses including sorghum (Brettell *et al.*, 1980) and ryegrass (Dale *et al.*, 1981).

Unlike the ability to form callus, the formation of embryogenic callus in *Miscanthus* also seemed to be controlled by other factors than the meristematic activity of the tissues. This is in agreement with investigations of other grass species (Morrish *et al.*, 1987). A high callus induction percentage in the present study was obtained from roots but no embryogenic callus was formed from this explant type. Basal sections of young leaves from greenhouse-grown shoots and immature inflorescences longer than 2.5 cm also showed high callus induction percentages but relatively low embryogenic callus percentages. An explanation could be that differences in endogenous levels of growth regulators like auxin, cytokinin and abscisic acid cause the variability in embryogenic callus formation as observed in different tissues and genotypes (Rajasekaran *et al.*, 1987a; Wenck *et al.*, 1988). Gradients in endogenous growth regulator levels along the callus inducing regions of leaves and immature inflorescences and their availability through the vascular system could explain the patterns of embryogenic callus formation obtained in this study.

Different 2,4-D concentrations had little effect on embryogenic callus formation. This is similar to results obtained for explants of perennial and Italian ryegrass (Creemers-Molenaar *et al.*, 1988), whereas in other grass species including maize (Pareddy & Petolino, 1990), barley (Thomas & Scott, 1985) and rye (Linacero & Vázquez, 1986), optimum 2,4-D concentrations for embryogenic callus formation have been found within the concentration range investigated in the present study. Differences in sensitivity to 2,4-D depend on species, genotype, explant type and developmental stage of the explant and have in some studies been related to the endogenous growth regulator level of the explants (Rajesekaran *et al.*, 1987a, 1987b; Carnes & Wright, 1988; Wenck *et al.*, 1988). The embryogenic callus formation on the different explant types at different developmental stages of *Miscanthus* was, however, independent of the 2,4-D concentrations. It is therefore likely that the different explants in the present study have a content of endogenous growth regulators that does not allow 2,4-D to further stimulate the embryogenic callus formation. In other investigations, exogenous addition of cytokinin or abscisic acid in combination with 2,4-D have been reported to increase the embryogenic callus percentage in some grasses (Bhaskaran & Smith, 1990) and the addition of cytokinin to the induction medium has been found to increase the percentage of shoot-forming callus in *Miscanthus* × *ogiformis* Honda 'Giganteus' (Petersen & Holme, 1992; Lewandowski & Kahnt, 1993).

In this study the embryogenic callus obtained from immature inflorescences of *Miscanthus* showed a higher regeneration capacity than the callus obtained from shoot apices or leaves. In many grasses immature inflorescences have been found to be well-suited for the initiation of callus and regeneration of plants through somatic embryogenesis (Brettell *et al.*, 1980; Tabaeizadeh *et al.*, 1990; Lewandowski & Kahnt, 1993). Since the immature inflorescence is the site of zygotic embryo development, the physiological and biochemical status of these explants might be more appropriate for embryo development and germination than explants from leaves and shoot apices. The physiological and biochemical status of explants appears to be very important for somatic embryogenesis (Morrish *et al.*, 1987; Vasil, 1987). In addition to the high regeneration capacity, the embryogenic competence of the callus obtained from immature *Miscanthus* inflores-

cences could be maintained by subculture and selection. The long-term embryogenic callus was used to establish embryogenic cell suspensions from which plants regenerated through somatic embryogenesis.

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