Establishment of an efficient *in vitro* culture and particle bombardment-mediated transformation systems in *Miscanthus sinensis* Anderss., a potential bioenergy crop

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Abstract

Plants belonging to the genus *Miscanthus* are considered promising bioenergy crops. Here, we report on the establishment of *in vitro* culture and particle bombardment-mediated transformation systems in *Miscanthus sinensis* Anderss.. Callus was induced efficiently from mature seeds in a medium containing a combination of a relatively high level of 2,4-dichlorophenoxyacetic acid (5 mg L^{-1}) and a relatively low level of 6-benzyladenine (BA) (0.01 mg L^{-1}). Callus production potential of 18 accessions of *M. sinensis*, which were collected from various sites in Japan, was compared. Significant correlation was detected between callus production frequency and average annual air temperature in collection sites. An accession from Tanegashima Island showed the highest production of compact (embryogenic) callus. We found that supplementing 2 mg L^{-1} BA in the culture medium was optimal for plant regeneration; higher levels of BA concentration allowed callus to turn brown whereas lower levels of BA kept the callus growing. Both compact and friable calli were suitable for particle bombardment transformation. Through selection under the presence of 50 mg L^{-1} hygromycin for 3 weeks and subsequently under the presence of 150 mg L^{-1} for 1 month, hygormycin-resistant calli survived, in which 72.2 % were fully fluorescent calli.

Polymerase chain reaction amplification of the surviving callus via this selection regime confirmed the presence of transgenes. This is the first report on the establishment of *in vitro* culture of *M. sinensis* originating in Japan, variation of callus formation among accessions collected from various sites, and particle bombardment-mediated transformation of callus.

Keywords

callus induction; *Miscanthus sinensis*; particle bombardment; regeneration; selection regime; transformation

Introduction

Plants belonging to the genus *Miscanthus* are perennial rhizomatous grasses with origins in Eastern Asia (Clifton-Brown *et al*., 2007). Since the 1960s, *Miscanthus* has been investigated the yield potential for cellulose fiber production in Europe (Scurlock, 1998). These plants are ranked among the most promising bioenergy crops (Carroll & Somerville, 2009). Among current potential sources of bioenergy, such as woody plant biomass, short-rotation coppice, agricultural waste materials and by-products from crops, and high-yielding grasses (Jakob *et al*., 2009), *Miscanthus* as biomass grass is considered a superior option (Smeets *et al*., 2009). The plants utilize the energy-efficient C_4 photosynthetic pathway (Naidu *et al*., 2003) which is considered to contribute to their bioenergy crop potentials, such as low-nutrient requirements (Lewandowski *et al*., 2003; Heaton *et al*., 2004), high water-use efficiency (Clifton-Brown *et al*., 2002), and high productivity (Clifton-Brown *et al*., 2001).

In the genus *Miscanthus*, *Miscanthus* ×*giganteus* Greef & Deuter ex Hodkinson and Renvoize, which is thought to be a spontaneous hybrid between *M. sinensis* Anderss. (Japanese common name: “susuki”) and *M. sacchariflorus* (Maxim.) Hack (Japanese common name: “ogi”) (Linde-Laursen, 1993; Greef *et al*., 1997), has

generated considerable interest for its high biomass yield; recent reports showed a maximum yield of 61t ha^{-1} (Heaton *et al.*, 2008). However, because of lack of fertility, propagation of *M.* × *giganteus* plants has to be done by planting rhizome fragments (Clifton-Brown *et al*., 2001; Christian *et al*., 2005). In addition, only a single genotype of *M.* × *giganteus* is available. These features are constraints on the use of *M.* × *giganteus* as a material for breeding and improvement. Meanwhile, *M. sinensis* can be used as a breeding material given its fertility and abundant genetic diversity (Stewart *et al*., 2009). It is also known that selections of *M. sinensis* have biomass yields comparable to that of *M.* × *giganteus* in some experimental sites, such as North Europe (Christian *et al*., 2005). It is also capable of being established by seeds.

Genetic transformation can be expected to be applied on *Miscanthus* to produce genetically modified plants with desired characters. One of the targets of improvement of *Miscanthus* is to change the content and optimize the ratio of lignin, cellulose, and hemicelluloses in cell walls (Hisano *et al*., 2009). This is one of the most popular approaches for improvement of saccharification which is important for bioenergy crop because high saccharification benefits fermentation of sugars into ethanol (Li *et al*., 2008; Hisano *et al*., 2009). Postponing or eliminating flowering, modifying plant height, tiller number and density, and stem thickness are considerable challenges for

maximization of biomass yield (Jakob *et al.*, 2009). In addition, improving the tolerance to biotic and abiotic stresses can protect biomass production from environmental infections.

For breeding purposes, such as genetic transformation, chromosome double and *in vitro* propagation, to further optimize *Miscanthus* as bioenergy crop, tissue culture on *M.* × *giganteus* has been successful in this genus (Petersen, 1997; Petersen *et al*., 1999; Holme & Petersen, 1996; Holme *et al*., 1997). In *M. sinensis,* pollen culture has been established recently for the purpose of haploid production (Głowacka & Jeżowski, 2009). Although young inflorescences are ideal explants for callus production (Głowacka *et al*., 2010; Głowacka & Jeżowski, 2009), influorescences can be obtained only in a limited period of the year. To prepare sufficient amount of calli that can be used for genetic transformation, season-independent materials, such as mature seeds, are needed.

The efficiency of cell propagation by *in vitro* culture often depends on genotypes. Accordingly, it is important to screen a wide range of accessions collected from various sites in terms of the response to *in vitro* culture. In one of the centers of diversity of *Miscanthus*, Japan, *M. sinensis* is distributed throughout the country from the northern island of Hokkaido to the subtropical Ryukyu Islands (Koyama, 1987); native

populations of *M. sinensis* contain a high level of genetic variation (Stewart *et al*., 2009).

Despite its importance as a bioenergy crop, neither material suitable for tissue culture nor transformation methods has been reported for *M. sinensis.* The objectives of this study were to establish an efficient *in vitro* culture and plantlet regeneration in *M. sinensis.* We also compared the ability to induce callus using several *M. sinensis* accessions. In addition, we optimized selection conditions of transformed cells following particle bombardment using the green fluorescent protein (*gfp*) and hygromycin (Hm) phosphotransferase (*hpt*) genes as selectable markers, and consequently established a method for transformation of cultured cells in *M. sinensis*.

Materials and methods

Seeds sterilization

Eighteen accessions of *M. sinensis* in the present study were collected in Japan where the latitudinal range from 30.5°N (Tanegashima) to 44.2°N (Tomamae) (Table 1). High-quality seeds were surface sterilized in sodium hypochlorite (1% active chlorine)

for 1.5 hrs followed by three rinses in sterile distilled water. The seeds were then kept for 24 hr at 4°C and sterilized again in the same way for 30 min.

Callus induction

For callus induction, MS basal medium (Murashige & Skoog, 1962) containing 30 g L^{-1} sucrose, which was supplemented with combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1, 5, 10 mgL^{-1}) with 6-benzyladenine (BA) (0, 0.01, 0.1 mg L^{-1}), was tested. Twenty-five seeds were placed on each callus-induction medium. Approximately 100 seeds of each line were tested for callus induction with three replicates. All media in this study were solidified with 2 g L^{-1} gelrite, adjusted to pH 5.8, and autoclaved. Callus was induced in darkness at 27 ± 1°C. The calli were subcultured at 1-month intervals in callus-subculture medium, labeled as MIS-S meidum, which is MS basal medium supplemented with 30 g L^{-1} sucrose, 1.5 mg L^{-1} 2,4-D and 0.01 mg L^{-1} BA.

Plant regeneration

To regenerate shoots from callus, MS basal media containing 30 g L^{-1} sucrose and

different concentration of BA (0, 0.1, 0.5, 1, 2, 4 mg L^{-1}) were compared. Compact calli of accession JM0138 (Tanegashima; Table 1) were divided into 5-mm diameter pieces. Four pieces were used in each type of medium with 3 replicates. The number of shoots was counted one month later. Shoots were placed in half-strength MS medium to develop roots. The regenerating shoots were incubated at a photoperiod of 16 hr light/8 hr dark, 26±1°C.

Plasmid DNA for particle bombardment

Plasmid pAcH1 containing the *hpt* gene and plasmid pUC19-GFP containing the *gfp* gene (Niwa *et al*., 1999) were co-bombarded. The plasmid pAcH1 was described in Takahashi *et al*. (2005). The *gfp* gene with viral Cauliflower Mosaic Virus 35S (CaMV 35S) promoter and nopaline synthase terminator was digested with *Hind*III and *Eco*RI from pMDC123-GFP (Sato *et al*., 2007). This cassette was cloned into pUC19 to create the reporter gene vector pUC19-GFP.

Particle bombardment was implemented by a device IDERA GIE-III (Science TANAKA, Ishikari, Hokkaido, Japan) as the manufacturer's instructions. Twenty pieces of 5-mm-dia. callus which were freshly subcultured in MIS-S medium for 10 to

14 days were placed in the center of an osmotic medium plate (MIS-S medium supplemented with 0.3M sorbitol and 0.3M mannitol) for 6-8 hours before bombardment. Optimal bombardment device parameters, such as bombardment distance of 5 cm, gas pressures of 5 kg cm^{-2}, and vacuum at 700 mm Hg, were adopted based on the number of green fluorescence spots 2 days after bombardment in the preliminary experiment. Three callus types (compact, friable and soft) were tested for the ability to be transformed.

Selection regime

Calli were kept in osmotic medium for 24 hr after bombardment and then transferred to MIS-S medium. Two days after bombarding, transient expression was observed using a florescence microscope (Keyence, VB-G25 series, Japan). After an additional 4-5 days of culture in MIS-S medium, callus was divided into four to five 3-mm-dia. pieces and transferred to MIS-S medium supplemented with 50 mg L^{-1} Hm for the first round selection. The concentration of Hm was decided by the preliminary observation that non-transgenic callus could not survive when more than 50 mg L^{-1} of Hm was present. After three weeks of selection, Hm-resistant calli were divided into four to five

3-mm-dia. pieces (3 mm in diameter) to undergo the second round selection. MIS-S media supplemented with Hm of 100 mg L^{-1} or 150 mg L^{-1} were texted in the second round selection process. Thirty callus pieces were placed in each plate with three replicates.

Molecular analysis of transgenic callus

Total DNA was isolated from putative transgene-containing calli by the CTAB method (Doyle & Doyle, 1987). Polymerase chain reactions (PCR) were carried out in 20 μl volumes using *rTaq*TM (TaKaRa, Tokyo, Japan). Primers (5′ GCTGGGGCGTCGGTTTCCACTATCCG 3′ and 5′ CGCATAACAGCGGTCATTGACTGGAGC 3′) designed to amplify 375 bp *hpt* fragment. Two hundred to 300 ng genomic DNA was included in the reaction mixture. The PCR reaction was started with 94^{o}C for 5 min and then 30 cycles of 95^{o}C for 30 s, 62^{o}C for 40 s, and 72^{o}C for 30 s, followed by the final extension at 70^{o}C for 7 min. Primers (5′ CACATGAAGCAGCACGACTT 3′ and 5′ ACTGGGTGCTCAGGTAGTGG 3′) were used for 385 bp *gfp* gene fragment amplification. The PCR conditions for the amplification of the *gfp* gene fragment were

the same as those for the *hpt* gene fragment except that annealing was done at 56^{o}C.

Results and discussion

Callus induction

In tested callus-induction media, soft, transparent callus was initially induced from seeds. Compact embryogenic callus was then induced from soft callus (Fig. 1). The induction of callus was affected by concentrations of 2,4-D and BA in the medium. Callus was induced at a slightly higher frequency in the medium containing 5 mg L^{-1} 2,4-D than that containing 1 mg L^{-1} or 10 mg L^{-1} 2,4-D in absence, or presence of 0.01 mg L^{-1} or 0.1 mg L^{-1} BA (Fig. 2; open bars). A medium containing 5 mg L^{-1} 2,4-D has also been used for callus induction in various grass species (Jensen *et al*., 2004; Altpeter *et al*., 2000; Hisano *et al*., 2004; Wang *et al.*, 2001; Takahashi *et al*., 2005; Bai & Qu, 2001). When medium was supplemented with 5 mg L^{-1} 2,4-D, BA promoted the production of compact callus. In the presence of 5 mg L^{-1} 2,4-D without BA, there was 50% callus production (Fig. 2; open bars), however, in which only 3.1% frequency of compact callus (Fig. 2; filled bars). The medium with 5 mg L^{-1} 2,4-D and 0.01 mg L^{-1}

BA produced compact calli at a significantly higher frequency (22.9%) (p<0.05) (Fig. 2; filled bars). Based on these results, MS basal medium supplemented with 5 mg L^{-1} 2,4-D and 0.01 mg L^{-1} BA (named MIS medium) was the optimal medium for callus induction.

Cooperation of auxin and cytokinin is common in plant embyogenic potential induction (KrishnaRaj & Vasil, 1995). Also, a low level of cytokinin often plays an important role in embryogenic callus development (Raghavan, 1989). In our study, cytokinin BA was also proven to be a regulator of embryogenic callus formation in *M. sinensis*. We also tested other concentrations of BA (i.e., 0.2 and 0.5 mg L^{-1}), which have been used in many grass species (Chai *et al*., 2002; Toyama *et al*., 2003; Luo *et al*., 2004; Dalton *et al*., 1998; Sato & Takamizo, 2006; Dai *et al*., 2003; Kumar *et al*., 2005). However, in *M. sinensis*, the induction frequency of callus in the presence of 0.2 or 0.5 mg L^{-1} BA was lower than in the presence of 0.01 mg L^{-1} BA (data not shown).

After 4-5 weeks of culture for callus induction, compact callus was transferred and maintained by monthly subculturing in MIS-S medium. Along with the culture time, compact callus gradually turned to be friable and then to be soft. The property of compact callus was white, compact, and solid, with smooth surfaced globular structures. Friable callus was light yellow, friable, solid and glossy. Also, soft callus was soft,

translucent, and tarnish. Compact callus all turned soft or even watery. It also lost the ability to regenerate if it was left in the medium for over 1 month without any subculture.

Variation in the callus production ability of 18 accessions of M. sinensis

The average annual temperature in the collection sites was correlated with the efficiency of callus production in *M. sinensis* (Table 1). There was a tendency that the seeds collected in warmer regions could form higher percentage of compact callus ($r=0.862$, $p<0.001$) (Fig. 3). Accessions collected in warm and temperate regions displayed high callus formation under the present callus culture conditions of the study (i.e., 27^{o}C). This is consistent with the fact that incubating temperature at 26-28^{o}C is suitable for callus culture of warm season grass species (Toyama *et al*., 2003; Smith *et al*., 2002; Luciani *et al*., 2007). However, accessions from cooler sites may prefer a slightly lower temperature, e.g. 24^{o}C-25^{o}C, for callus induction such as cold-season grasses (Altpeter *et al*., 2000; Cho *et al*., 2000; Chai *et al*., 2004). Among all the accessions tested in this study, accession JM0138 derived from plants naturally grown in Tanegashima Island (Latitude 30.5^{o}N, average temperature 19.9^{o}C) was apparently superior in terms of

compact callus production (Table 1). Based on these results, we used this plant line as a material for the subsequent analyses.

Shoot regeneration

In regeneration media, a high BA concentration (i.e., 4 mg L^{-1}) induced browning of callus, and a low BA concentration (i.e., 0.1 mg L^{-1}) did not induce any change in morphogenesis. An optimum concentration of BA, which would not induce browning of callus, prevent callus from over propagation, or allow callus to regenerate, was needed. Based on the observed frequency of shoot formation after 1 month of culture in the regeneration media, 2 mg L^{-1} BA was found to be most suitable for regeneration in *M. sinensis* (Table 2, Fig. 4). However, it also observed that 2 mg L^{-1} BA led to callus browning and death at a low frequency. Other cytokinins, such as kinetin (Montoro *et al*., 1993; Lin *et al*., 2004) and thidiazuron (Lin *et al*., 2004) were also observed leading to browning of callus. We found that transferring the non-browning part of callus into a fresh medium was effective to avoid these problems (data not shown).

Callus types suitable for particle bombardment

We counted the number of GFP-expressing spots on calli after they were bombarded with plasmid containing the *gfp* gene (Fig. 5). The frequency observed in experiments using compact or friable calli was higher than that in experiments using soft calli, indicating that both compact and friable calli were more suitable for particle bombardment than soft callus ($p<0.001$) (Fig. 5). Embryogenic calli are generally suitable explants for transformation because the regenerable cells are not excessively shielded and can cover most of the target area of bombardment (Bower & Birch, 1992). We supposed that compact callus of *Miscanthus* was embryogenic by a similar description and image of "type 3" callus of sugarcane *(Saccharum* spp. hybrids) (Taylor *et al*., 1992), which is close to *Miscanthus*. In the same way, the friable callus here were supposed to be organogenic like the "type 4" of sugarcane callus, which was yellow, friable, and formed following the "type 3" one (Taylor *et al*., 1992). In this manner, the embryogenic and organogenic callus of *Miscanthus* were both accessible for particle bombardment.

Selection regime

Calli bombarded with particles coated with plasmids containing the selection marker gene were cultured in the medium containing Hm. We applied two rounds of selection in the presence of Hm. After the first round selection that was done in the presence of 50 mg/L Hm, 36.2±5.4% calli survived and 25.0±5.1% Hm-resistant calli showed green fluorescence. However, the green fluorescence appeared as just green spots and in most cases only a small part of the callus had green spots, which suggested that *gfp* expression occurred in small numbers of cells.

In the second round selection, we placed 30 callus pieces per plate in the medium containing 100 mg L^{-1} or 150 mg L^{-1} Hm. Previous studies suggested that a limited range of callus number in one plate was suitable for selection of transformed cells (Leelavathi *et al*., 2004; Wu *et al*., 2008). We have ever tested a selection with a callus number of 4 callus pieces per plate (data not shown) which was smaller than 30 callus pieces decided in the second round selection. Calli displayed green fluorescence spots and had portions lacking green fluorescence, indicating imperfect selection. Taking this into account, it was adopted that 30 callus pieces were in per plate. In MIS-S medium containing 100 mg L^{-1} Hm, 33.3% calli Hm-resisted (Fig. 6; open bars) and 17.0 % calli (Fig. 6; filled bars) were fully fluorescent, i.e., 52.0% of Hm-resistant calli were fully fluorescent (17.0% divided by 33.3%); in the medium containing 150 mg L^{-1} Hm,

20.0% calli Hm-resisted (Fig. 6; open bars) and 15.0% calli were fully fluorescent (Fig. 6; filled bars), i.e., 72.2% of Hm-resistant calli were fully fluorescent (15.0% divided by 20.2%). That indicated that 150 mg L^{-1} Hm was more efficient than 100 mg L^{-1}. A half-strength MIS-S medium and a quarter-strength MIS-S medium supplemented with 100 mg L^{-1} or 150 mg L^{-1} Hm, were also tested, because reduction of the extra nutrition may limit the survival of non-transgenic cells. However, all calli died in these two media (data not show). Based on the results that 150 mg L^{-1} Hm in MIS-S medium could arrest the growth of non-transgenic cells while support the development of entirely fluorescent calli, we concluded that 150 mg L^{-1} in MIS-S medium was the optimal concentration of Hm in the second round selection. Through this selection, stable transgenic cells could continue to proliferate (Fig. 7).

According to the selection regime, co-bombardment of the *hpt* gene and *gfp* gene was done two more replicates. The number of callus was counted in the respective stages (Table 3). A total of 120 callus pieces, as initial material for bombardment, were divided into 522 callus pieces after 4-5 days of culture without Hm, and then subjected to the first round selection in the presence of Hm. 166 calli survived; 113 of them were *gfp* expressing calli. The *gfp*-expressing calli were further divided into 697 calli, and were subjected to second round selection. We found that 158 of them survived. Also,

101 out of the 158 calli had GFP fluorescence in the entire area.

Detection of transgene by PCR

Amplification of both the *hpt* gene and *gfp* gene occurred (Fig. 8), suggesting that these genes are maintained during the proliferation of the calli.

Conclusion

*Miscanthu*s spp. is one of the most intensively investigated bioenergy crops. Because of the limited genetic variation of the sterile hybrid *M.* × *giganteus*, increasing more attention is being given to one of its progenitors, *M. sinensis*. We established *in vitro* culture in *M. sinensis* collected from various sites in Japan for the first time and examined variation of callus formation ability using accessions. A particle bombardment-mediated method successfully delivered foreign genes into *M. sinensis* and the selection regime was optimized. From a total of 120 callus pieces, 101 calli appeared to contain both the *hpt* and *gfp* genes. The protocols established in the present study will be applicable to the production of genetically modified *M. sinensis* plants.

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Figure legends

Figure 1. Representative image of soft and compact calli. In callus induction media, soft, transparent callus initially developed, and compact callus was then induced from soft callus.

Figure 2. Combinatory effect of 2,4-D and BA on callus induction. Frequency of seeds that produced calli or campact calli in the presence of 2,4-D and BA at different combinations of concentration is shown. *Error bars* indicate the standard deviation obtained from three replicates.

Figure 3. Relationship between temperature in colleting sites of accessions and callus production ($r=0.862$, $p<0.001$). The temperatures are the average of five years from 2005-2009.

Figure 4. Representative image of shoots regenerated in the regeneration medium containing 2 mg L^{-1} BA after one month of culture.

Figure 5. Effect of compact, friable and soft callus types on transient *gfp* expression. The three types of callus were formed during callus subculture. *Error bars* indicate the standard deviation of three replicates of measurement.

Figure 6. Effect of Hm on callus survive or fully fluorescent callus formation. Percentage of survived callus or fully fluorescent callus in presence of 100 mg L^{-1} or 150 mg L^{-1} Hm in the second round callus selection. *Error bars* indicate the standard deviation obtained from three replicates.

Figure 7. Representative image of the callus with fluorescence in the entire area survived in the medium containing 150 mg L^{-1} Hm after the second round of selection. .

Figure 8. PCR analysis of *hpt* and *gfp* genes in calli survived after the second round selection. P, plasmid DNA control; Non, nontransgenic callus; C1 and C2, callus with GFP fluorescence.

Tables

Table 1. Origin of 18 accessions of *M. sinensis* used in the present study and their ability of compact callus production.

Accession	Collecting site	Latitude ^{o}N	Average temperature ^{o}C [a]	Compact callus (%)±SD
JM0001	Tomamae	44.2	7.4	10.0±1.19
JM0034	Ishikari	43.2	7.5	5.3±2.42
JM0040	Sapporo	43.0	8.5	2.7±0.72
JM0053	Yakumo	42.2	7.8	4.0±0.94
JM0057	Imakane	42.5	7.3	11.3±0.72
JM0062	Hakodate	41.7	8.8	13.3±0.72
JM0065	Hokuto	41.8	8.8	8.3±0.54
JM0067	Matsumae	41.4	10.3	5.0±0.94
JM0076	Shikabe	42.1	8.3	8.0±0.72
JM0093	Nobeyama	36.1	6.7	2.0±0.47
JM0094	Matsubarako	36.1	6.7	4.0±0.72
JM0095	Ooizumi	35.9	10.7	5.0±0.72

JM0099	Nagasaka	35.8	10.7	2.7±0.47
JM0122	Kochi 1	33.6	17.5	21.0±3.81
JM0123	Kochi 2	33.6	17.5	19.7±1.66
JM0125	Miyakonojo	31.5	16.1	22.7±2.94
JM0134	Takachiho	32.4	14.2	17.7±1.25
JM0138	Tanegashima	30.5	19.9	25.0±2.62

[a] The average annual air temperature (2005-2009). Downloaded from Japan Meteorological Agency.

Table 2. The number of calli generated shoots and the number of shoots in various regeneration media with different BA concentration.

BA concentration	Callus for regeneration (each replication)	Number of calli that generated shoots	Total number of shoots ± SD
0	4	1.3	3.3±1.2 [cb]
0.1	4	0.3	0.3±0.6 [c]
0.5	4	1.0	9.0±1.7 [ab]
1	4	2.0	8.0±2.6 [ab]
2	4	1.3	13.7±4.0 [a]
4	4	1.7	10.0±5.3 [ab]

The experiment was repeated for three times

Different letters indicate significant differences at $p<0.05$.

Table 3. The number of calli during the process of particle bombardment followed by two rounds of selection.

Experiment	Calli used for bombardment	Calli subjected to first round selection	Hm-resistant calli after first round selection (*gfp* expressing calli [a])	Calli subjected to second round selection [b]	Hm-resistant calli after second round selection (*gfp* expressing calli [c])
1	60	271	78 (49)	327	72 (49)
2	60	251	88 (64)	370	86 (52)
Total	120	522	166 (113)	697	158 (101)

[a] *gfp* green fluorescence spots expressing calli

[b] only *gfp* expressing calli were transferred for the second round selection

[c] calli with GFP fluorescence

Fig 1

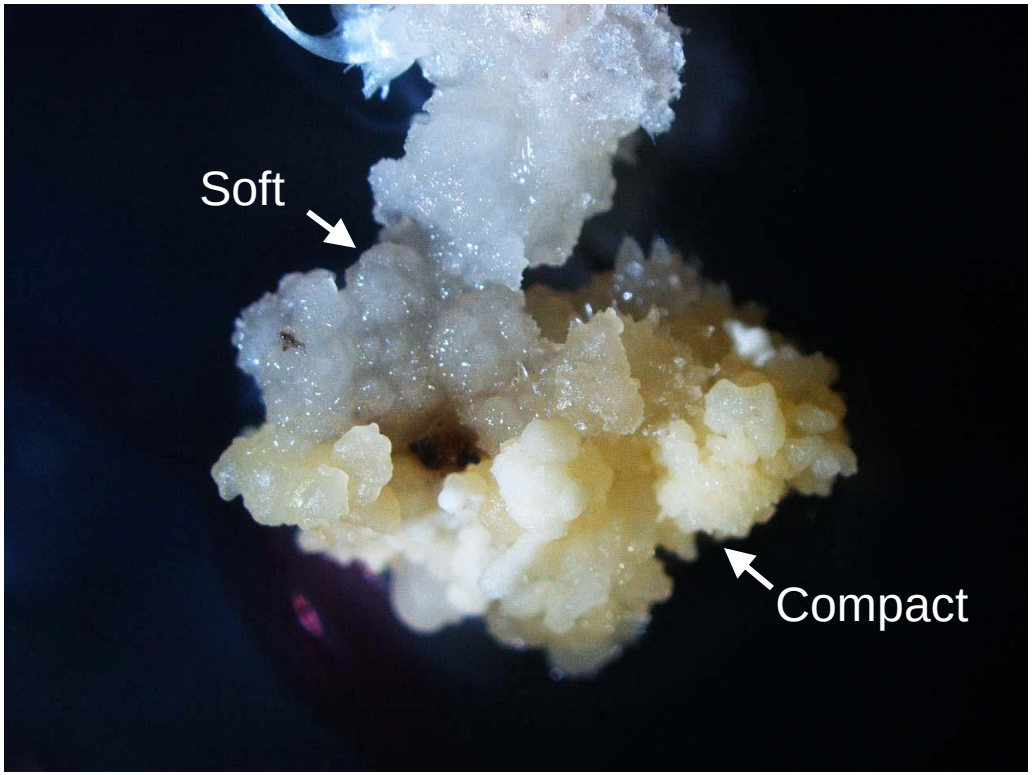

Fig 2

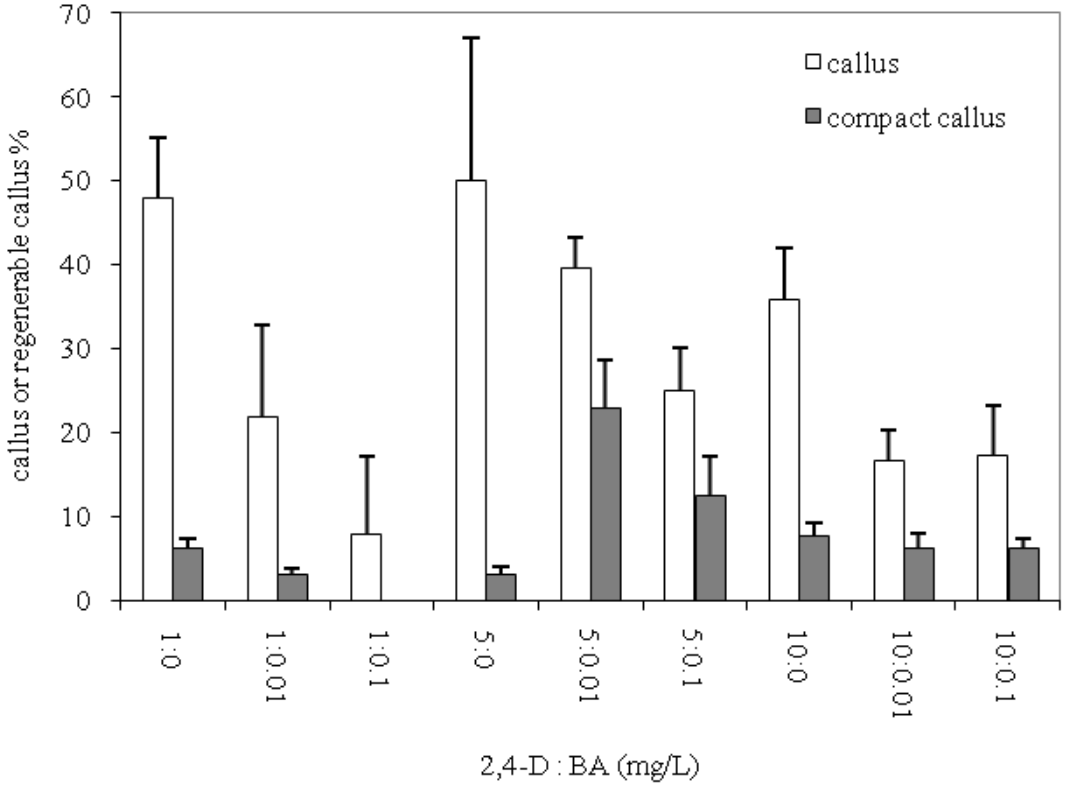
70
60
50
40
30
20
10
0
callus or regenerable callus %
callus
compact callus
1:0
1:0.01
1:0.1
5:0
5:0.01
5:0.1
10:0
10:0.01
10:0.1
2,4-D : BA (mg/L)

Fig 3

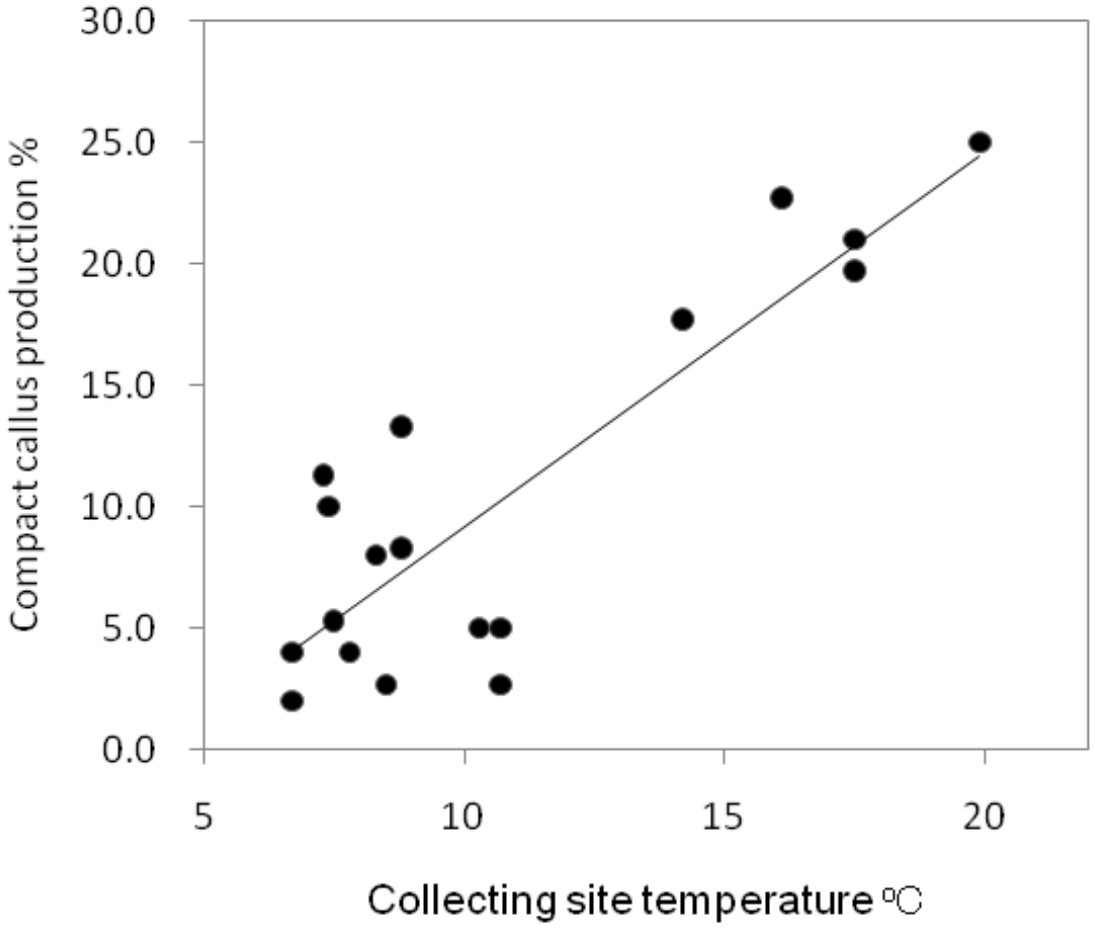
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Compact callus production %
5
10
15
20
Collecting site temperature ºC

Fig 4

Fig 5

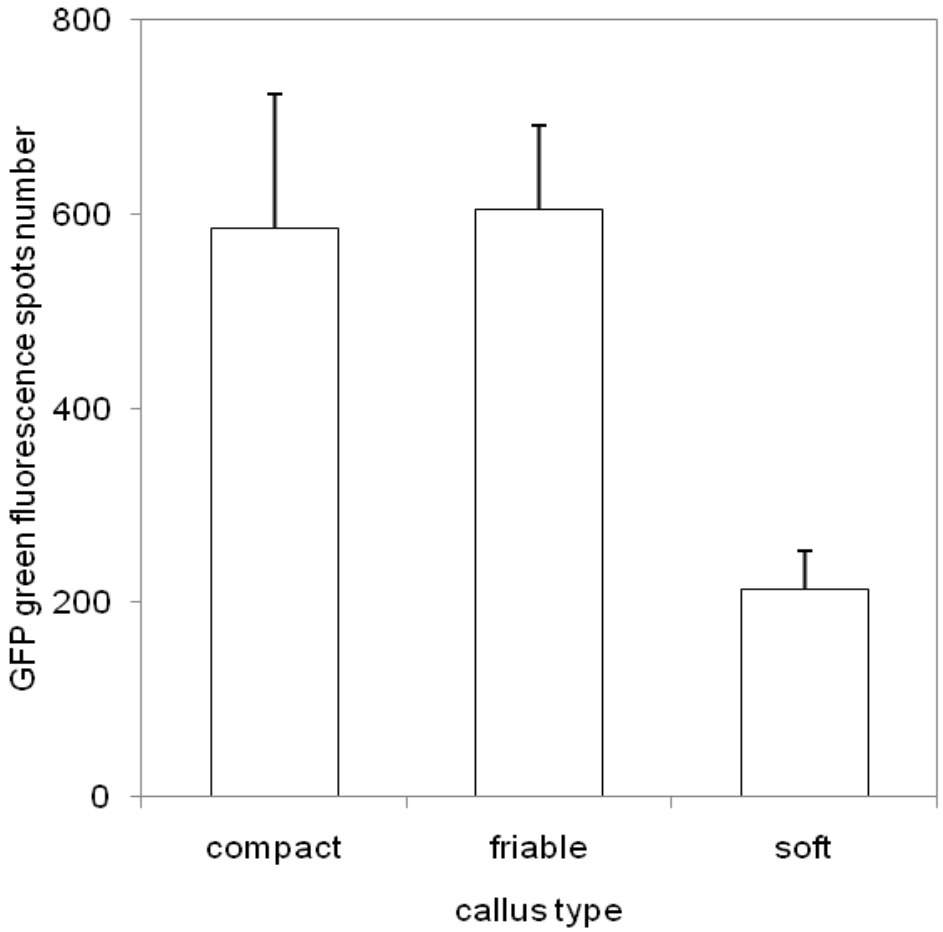
800
600
400
200
0
GFP green fluorescence spots number
compact
friable
soft
callus type

Fig 6

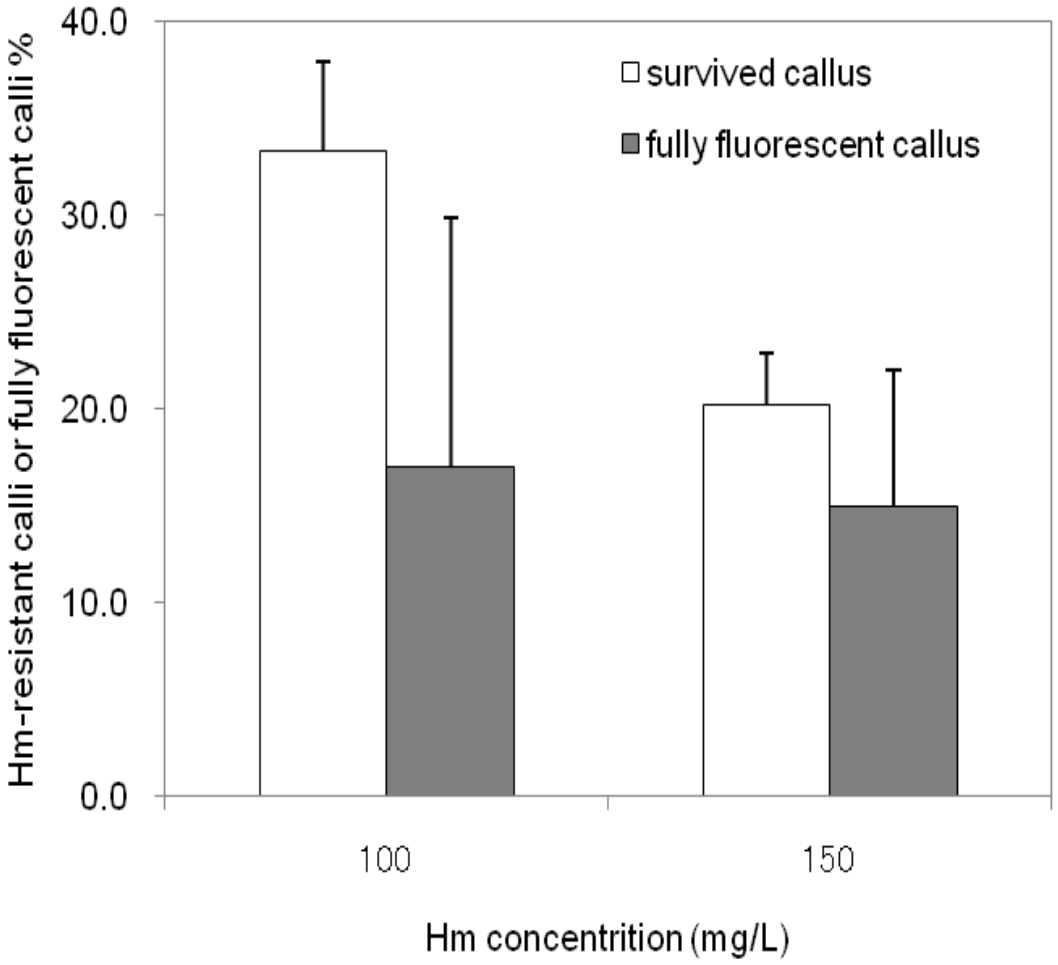
40.0
30.0
20.0
10.0
0.0
Hm-resistant calli or fully fluorescent calli %
survived callus
fully fluorescent callus
100
150
Hm concentrition (mg/L)

Fig 7

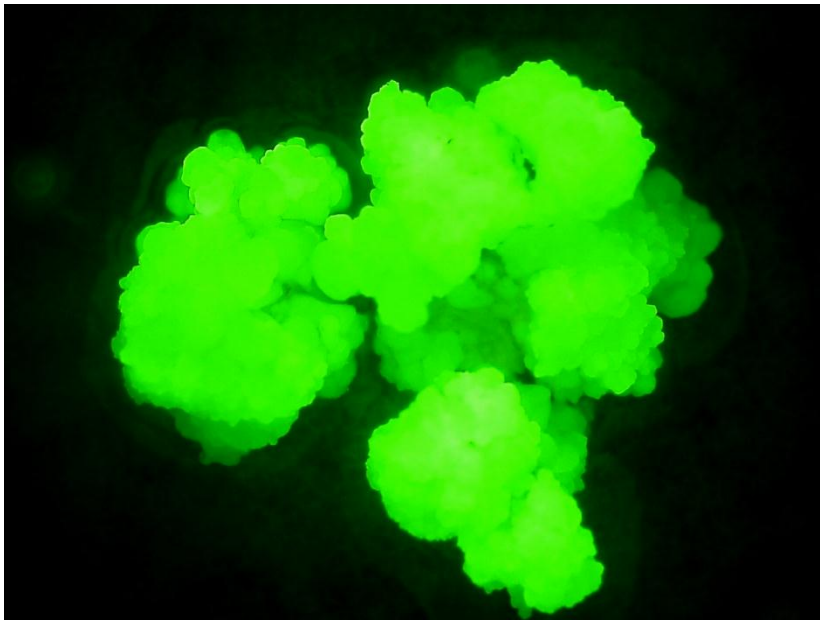

Fig 8