Rooting in Km selective media as efficient *in vitro* selection method for sunflower genetic transformation

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Abbreviations: *A*: agroinfected
AIM: Agrobacterium inducing media
BAP: benzyl amino purine
C: controls
CC: co culture
CTAB: Cetyltrimethylammonium bromide
Km: kanamycin
MS: Murashige-Skoog media
NAA: naphthalene acetic acid
OD: optical density
PCR: polymerase chain reaction
Re: Regenerating media
Tmt: timentine

Despite of numerous publications in sunflower genetic transformation, there is no efficient or reproducible protocol with low number of escapes. The latter would indicate that the selection method is not effective. In this work we used Km as selective agent, *Agrobacterium tumefaciens* EHA105 strain and a vector with the *nptII*

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gene under the *nos* promoter and *uidA* gene under *35S* promoter. The response of agroinfected (*A*) and control (*C*) explants during the *in vitro* culture was studied and in both cases in presence or absence of Km in order to assign a differential morphologic response between transformed and non-transformed plants. The characteristics analyzed were: height, colour/aspect of the plantlets, *in vitro* rooting and *in vitro* bud-flower development. Selection was applied from the third regeneration media. Among the *A* plantlets two were capable of rooting, being positive by PCR, whereas the *C* were unable to root in presence of Km. One of them gave 6 seeds and in these plants, it was determined the presence of the transgene by PCR and GUS staining. This work shows that in Km selection, colour/aspect of shoots is not useful as selection criteria whereas rooting is an effective selection method in which no escapes were obtained.

Transformation efficiencies have very low values, between 0.01 and 5%, so during the regeneration of transgenic plants, it is very important to set conditions where non-transformed cells or tissues are unable to grow and/or develop. This is even more important in the case of sunflower because *in vitro* regeneration of plantlets (T0) is via organogenesis, producing chimeras that not necessarily will give an offspring (T1) that carries and/or express the transgene, making the PCR analysis of the T0 not conclusive. For this reason, a selection gene is introduced along with the gene of interest, conferring the transformed cell the possibility to survive and regenerate in the presence of the selective agent (antibiotic or herbicide) whereas the non-transformed cells die or are unable to grow. Thus, the proper selection of a selective gene determines the possibility of success in the production of transgenic plants. Despite several publications in sunflower genetic transformation (Hewezi et al. 2002; Weber et al. 2003), there is no efficient or reproducible protocol with a low number of escapes, indicating that the selection method using Km is not effective. The aim of this work was to establish the use of the antibiotic Km as an efficient selective agent, trying to relate transgene presence with the differential *in vitro* behaviour.

MATERIALS AND METHODS

Seeds from the public genotype HA89 were used as plant material.

Agrobacterium tumefaciens EHA105 strain (Hood et al. 1993) was used, carrying a GUS-intron plasmid (Vancanneyt et al. 1990) as a transformation vector. Seeds previously disinfected, peeled and germinated for 24 hrs in MS1/2 media, were cut in half, resulting in 2 hemiembryos each one with a complete cotyledon. The cut was done inside a Petri dish with 20 ml of bacteria (OD_{660}: 0.05) grown in AIM media supplemented with acetosyringone. A co-culture (CC media) was performed for 2-4 days in

darkness at 22ºC. *C* explants were treated in the same way as *A* but co-culture treatment was performed in absence of *Agrobacterium*. For the regeneration step explants were transferred every 10/14 days through different regeneration media (Re1 to Re4) containing the antibiotic Tmt 0.3 mg/ml to inhibit *Agrobacterium* growth, as was previously determined in our laboratory. Finally, a passage to a rooting media for a 20 days period was made. *In vitro* conditions were 22-24ºC of temperature and 16 hrs light. Plantlets that showed a suitable radicular development were transferred to the greenhouse. Media formulation was based in Murashige and Skoog (1962). MS1/2: half saline concentration. All Re media contained: $AgNO_3$ 0.82 µg/l and Tmt 0.3 mg/ml. Re1: BAP 0.2 mg/ml and NAA 0.01 mg/ml. Re2: BAP 0.2 mg/ml. Re3: BAP 0.1 mg/ml. Re4: no hormones. RA (rooting): NAA 0.1 mg/ml and Tmt 0.3 mg/ml.
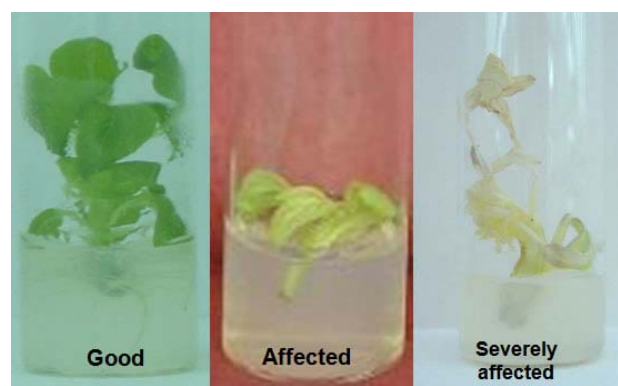

Figure 1. Shoots showing the three different color/aspects in which plantlets were classified: good, affected and severely affected.

Selection was performed from Re3; shoots *C* and *A* were divided in media with or without Km (50 mg/l). Note was taken from the phenotype of the shoots. Shoots that rooted in RA media with Km were transferred to the greenhouse.

χ^2- Chi square statistical analysis was performed.

GUS-intron gene expression was demonstrated by histochemical staining assays (Jefferson et al. 1987). Genomic DNA extraction was performed using the CTAB method (Saghai-Maroof et al. 1984). PCR was performed using Invitrogen Taq polimerase (1 U) and 0.1 µM of each primer (GUS391 UP: 5'-ACC GTT TGT GTG AAC AAC GA-3' and GUS2: 5'-GGC ATT GGT TTC GAA GCG GG-3'). Negative controls: mix without the addition of DNA and other with DNA from a non-transformed plant. Positive controls: GUS-intron plasmid DNA and DNA from *Solanum tuberosum* transformed with the *uidA* gene.

RESULTS AND DISCUSSION

A total of 724 plantlets were analyzed in 4 different culture situations: non-agroinfected controls (*C*), non-agroinfected controls treated with Km (*C-Km*), agroinfected (*A*) and agroinfected treated with Km (*A-Km*). In order to study the

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in vitro response to the antibiotic and determine if there were differential responses of the cultures in the 4 analyzed situations the following four parameters were studied:

- *In vitro* rooting: This is an important tip as root development in the *in vitro* shoot avoids the laborious, time-consuming and not always successful grafting when shoots are transferred to greenhouse conditions. We have previously established a protocol for *in vitro* rooting with a success level of 60%.

- Height: Data from shoot height were grouped in three categories, shorter than 2.5 cm, between 2.5 and 3.5 cm and taller than 3.5 cm.

- Floral-bud development: This is an unwanted parameter as its presence interferes and makes more difficult the transference to *ex vitro* conditions, so if this condition was favored by the selection method this would not be very useful.

- Colour/aspect: These data were also grouped in three categories, *good*: shoots with an intense green colour and healthy aspect, *affected*: the green colour was not so intense and sometimes some leaves showed certain abnormal shape or texture, *severely affected*: a clear unhealthy aspect, shoots with very pale green or white colour and sometimes severe changes in leave shape and general aspect, (Figure 1).

The results obtained are shown in Table 1. No significant differences were observed among the 4 analyzed treatments in none of the four studied parameters but interestingly no *C-Km* from 233 shoots was able to develop roots while in the group of *A-Km*, 2 shoots from 282 (0.7%) developed *in vitro* roots. These two shoots were transferred to the greenhouse and only one of them produced seeds (6 seeds) allowing the analysis of the T1 filial.

The molecular analysis was done by PCR using the GUS-intron probes on the DNA of the following: a) 199 *in vitro*

shoots from *C, A* y *A-Km*, b) leaves from the two plants transferred to the greenhouse that rooted *in vitro* in the presence of Km and c) leaves from the six plants grown in the greenhouse of the T1 filial. It must be taken into account that plantlets from a) and b) are chimeras. The PCR results, presence or absence of the transgene, of the *A-Km* shoots are summarized in Table 2.
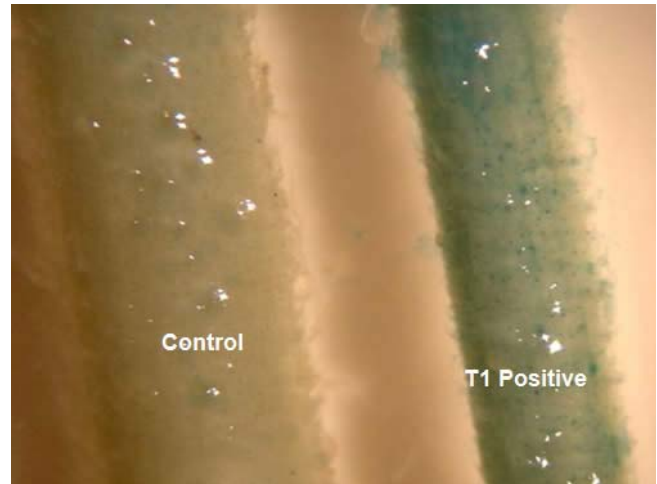

Figure 2. Activity of the β-glucuronidase enzyme in stems from control and T1 plants grown in the greenhouse.

In order to find a relation between the molecular results and the observed *in vitro* shoot response, data from the *A-Km* shoots shown in Table 1 and in Table 2 were analyzed and compared. The statistical χ^2 analysis (p-value 0.1) revealed that there was no association between transgene presence and a given colour/aspect, height or the presence of floral-buds. In relation to *in vitro* rooting, as the number of rooted shoots was rather low no statistical difference was observed. Nevertheless, it must be highlighted that both rooted shoots in Km resulted positive by PCR analysis and that no control shoot could root in presence of the selective agent (Table 2). The T1 plants were studied by PCR and by histological GUS assay (Figure 2), resulting all of them positive.

In previous sunflower transformation studies using Km selection for hemiembryos transformation at the same

Table 1. Percentage of shoot frequency grouped by height, color/aspect, floral-bud and *in vitro* rooting, according to the four analyzed characteristics.

		C	*C-Km*	*A*	*A-Km*
Height (cm)	Lower than 2,5	57,3%	57,7%	23,6%	53,5%
	2,5-3,5 11%		21,2%	29,9%	24,5%
	Taller than 3,5	31,7%	21,2%	46,5%	22,0%
Color/Aspect	Good 85,4%		59,6%	72,4%	67,4%
	Affected 11,0%		25,0%	21,3%	21,6%
	Severely affected	3,7%	15,4%	6,3%	11,0%
Floral-bud	No 74,4%		86,5%	63,0%	88,7%
	Yes 25,6%		13,5%	37,0%	11,3%
Rooting	No 61,0%		100%	38,6%	99,3%
	Yes 39,0%		**0%** 61,4%	**0%**	**0,7%**

concentration that was used in this work, Knittel et al. (1994) found that the selection procedure induced discolouring of the sensible sectors while other resistant areas stayed dark green. The authors mentioned that these areas were transferred to a rooting media, obtaining 7% efficiency but no data on the escape level was presented. Malone-Schonenberg et al. (1994) reported a 20% escapes on selecting with 200 mg/l Km, and this level increased with 25 mg/l Km. Burrus et al. (1996) using two kinds of explants (complete apical embryonic meristems or half-cut) and 50 mg/l Km, observed 18% of escape while Lucas et al. (2000) reported a 72%. In order to avoid grafting difficulties, Hewezi et al. (2002), treated good colour and healthy shoots with a rooting solution in their base and the level of escapes was of 10%. On the other hand, Müller et al. (2001), performing a selection on regenerated shoots from a non-meristematic tissue with 1, 3, 10 and 15 mg/l Km, were not able to select transformed shoots and identify the transgenic events by Southern or Western analysis.

In the present study the results obtained showed that the colour and aspect of the shoots are not valid physiological parameters for identification of transgenic events as 67.4% of the *A-Km* putative transformed plantlets according to these parameters are escapes (Table 1). Furthermore, 85.4% of the shoots from the non-transformed group (PCR -) presented a healthy aspect, so they would have been selected as putative transgenic (Table 2). In this way, it is shown here that there is no association between shoot colour and the presence of the transgene, therefore, this physiological condition must not be considered as a selection criterion in sunflower transformation. There is only one article with a different selection criterion, Grayburn and Vick (1995) reported that non-transformed shoots are unable to develop roots if they were cultured previously in a Km regenerating media. Up to now, our work is the first one where the selection criterion is the *in vitro* root development in presence of Km.

Table 2. Percentage of positive (PCR +) and negative (PCR -) shoots for the GUS-intron transgene presence.

		PCR +	PCR -
Height (cm)	Lower than 2,5	64,8%	66,7%
	2,5-3,5 27,8%		27,1%
	Taller than 3,5	5,6%	6,3%
Color/Aspect	Good 74,1%		85,4%
	Affected 25,9%		14,6%
	Severely affected	0%	0%
Floral-bud	No 75,9%		83,3%
	Yes 24,1%		16,7%
Rooting	No 96,3%		100%
	Yes	**3,7%**	**0%**

CONCLUDING REMARKS

The selection by *in vitro* rooting in a Km culture medium is a promising successful method as no escapes were obtained and the transformation efficiency was of 0,7%. Another interesting conclusion is that shoot colour must not be considered as a selection criterion when Km is the selective agent in sunflower transformation.

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