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Evaluation of soybean cultivars on the embryogenic and organogenic potential

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ABSTRACT. The first step in plant transformation using somatic embryogenesis or organogenesis for plant regeneration *in vitro* has been the identification of genotypes with the best totipotency. Thus, the objective of this experiment was to evaluate the totipotency of ten cultivars and one soybean line from the Coodetec Gene Bank. The somatic embryos developed from immature cotyledons measuring from 4 to 6 mm in length, and the organogenesis was evaluated from mature soybean seeds. The cultivars CD 201, CD 216 and CD 220 were the most promising genotypes to produce somatic embryos, whereas the cultivar CD 217 had the highest quantity of differentiated explants, which represents the best regeneration efficiency for participating in organogenic systems. In the experiment with somatic embryogenesis, time to plant regeneration was ten months, but the plants were regenerated after only four months in the experiment with organogenesis.

Keywords: *Glycine max*, somatic embryogenesis, organogenesis.

RESUMO. Avaliação de cultivares de soja quanto ao potencial embriogênico e organogênico. A identificação de genótipos que possuam maior totipotência quando submetidos à embriogênese somática e organogênese é a primeira etapa para a condução de um programa de transformação genética. Este trabalho teve como objetivo avaliar o potencial de embriogênese somática e organogênese em nove cultivares de soja e uma linhagem, do banco de germoplasma da Coodetec. Cotilédones imaturos de soja com 4 a 6 mm de comprimento foram utilizados para produção de embriões somáticos, induzidos com 2,4-D, e sementes maduras de soja foram usadas para avaliação do potencial organogênico. As cultivares CD 201, CD 216 e CD 220 mostram-se promissoras para produção de embriões somáticos, enquanto que para o sistema organogênico a cultivar CD 217 apresentou a maior quantidade de explantes diferenciados e manteve a maior eficiência de regeneração. Para obtenção de uma planta via embriogênese somática foram necessários 10 meses, em virtude do período necessário para obtenção dos explantes. O sistema organogênico se mostrou mais rápido, e plantas completas de soja foram obtidas em quatro meses de cultivo *in vitro*.

Palavras-chave: *Glycine max*, embriogênese somática, organogênese.

Introduction

Parallel to the traditional plant breeding, methods for enhancing the genetic variability has been developed and applied to all major crops. The establishment of these protocols is essential for increasing the number of superior genotypes. In soybean (*Glycine max* (L.) Merrill), for example, the cultivation of transgenic cultivars has been possible because of the several protocols in plant transformation.

Successful plant transformation consists of inserting DNA into target cells that must be capable of integrating this exogenous DNA, and these transformed cells must have effective participation in the germination tissue.

Complete transformation, however, requires a mechanism to develop the adult plants, and this mechanism requires *in vitro* protocols to regenerate these plants (PARROT; CLEMENTE, 2004). In soybean, fertile plants have been regenerated from cell or tissue cultures by using somatic embryogenesis or organogenesis mechanisms.

The main constraints to regenerate the transformed explants have been their levels of nutritional and hormonal requirements (DUSI, 1998). The continuous refinement of these

protocols has also been necessary when new genes are isolated, characterized and available for testing because numerous variables have to be adapted to the conditions of every laboratory. In the somatic embryogenesis of soybean plants, for example, research reports have showed the genotypic influence from donor plants (KOMATSUDA; OHYAMA, 1988; KOMATSUDA et al., 1992), type of explant, growth regulators (HARTWECK et al., 1988; BUCHHEIM et al., 1989), solidifying agents (FU; HUANG et al., 1995) and pH (KOMATSUDA; KO, 1990).

Most of the soybean genotypes with embryogenic potential are not yet well adapted to the field conditions, and they are easily overcome by newly elite cultivars. In transgenic breeding programs, therefore, gene transference from genotypes with embryogenic potential to these elite cultivars is necessary, and this transference has been made by plant crosses and selection.

Some reports have showed that plant regeneration by organogenesis depends less on the genotypes and, therefore, the organogenesis has been used in the routines of several laboratories (OLHOFT et al., 2003; OPABODE, 2006; PAZ et al., 2004; SHAN et al., 2005). Organogenesis, however, is a system much more complex because of the interactive effects from explant sources, growing media and environmental factors (GEORGE, 1993; JOY IV; THORPE, 1999; PIERIK, 1997). The organogenesis also depends on exogenous growth regulators as the auxins and cytokinins as well as tissue responses to hormonal changes during the cultivation period (SUGIYAMA, 1999).

Thus, the objective of this experiment was to select soybean cultivars and a line with potential to regenerate plants *in vitro* using somatic embryogenesis and organogenesis, and evaluate the regenerative efficiency of these genotypes.

Material and methods

Genetic material

The embryogenic and organogenic potential of nine soybean cultivars and one line from the Coodetec Gene Bank (CD 201, CD 202, CD 206, CD 211, CD 216, CD 217, CD 218, CD 220, CD 221 and the line CD 98-2644) were evaluated at the Tissue Culture Laboratory of the Cooperativa Central de Pesquisa Agrícola (Coodetec), Cascavel, Paraná State, Brazil.

Somatic embryogenesis

Soybean plants were cultivated under greenhouse conditions with photoperiod of 16 hours to obtain the cotyledonary explants. Cotyledons measuring from 4 to 6 mm in length were collected at the R_5 stage from healthy pods of vigorous plants. First, these pods were disinfested using 70% ethanol for 5 min. and 10% sodium hypochlorite (v v^{-1}) for 20 min. Thereafter, they were rinsed in distilled and deionized water to remove the excess of the sodium hypochlorite. The pods were opened to excise the cotyledon pairs which were abaxially faced on the culture medium.

Every genotype had ten cotyledons cultivated in Petri dishes, 90 x 15 mm (SANTARÉM et al., 1997). These Petri dishes were filled with 25 mL of the medium MSD40 (FINER; NAGASAWA, 1988) prepared with MS salts at half strength (MURASHIGE; SKOOG, 1962), B5 vitamin (GAMBORG et al., 1968), inositol (100 mg L^{-1}), 3% sucrose, 0.2% Phytagel, 180 μM 2,4-D and pH 7.0. Ten Petri dishes per genotype were incubated at 28 ± 1ºC for 30 days under the photoperiod of 16h and light irradiance of 25-30 μmol m^{-2} s^{-1} (SANTARÉM et al., 1997). The explants were subcultivated for more 15 days upon the development of the primary globular embryos. Thereafter, these globular embryos were isolated from regions close to the original explants and transferred onto the medium MSD20 to develop and multiply the embryogenic clusters. This growing medium was prepared with the MS (MURASHIGE; SKOOG, 1962) at half strength (WRIGHT, 1991), B5 vitamin (GAMBORG et al., 1968), inositol (100 mg L^{-1}), 3% sucrose, 0.2% Phytagel, 90 μM 2,4-D, and pH 5.8. The subcultures were also maintained in similar conditions during the induction period. During these steps, the embryogenic tissues were selected to choose embryos in globular stage. The proliferation period was extended for 6-8 weeks.

Embryos in the globular stage were cultivated in hystodifferentiation medium (MSM6AC) to obtain the cotyledonary embryos. This growing medium was prepared with MS salts (MURASHIGE; SKOOG, 1962), B5 vitamin (GAMBORG et al., 1968), 6% maltose, 0.5% active charcoal, 0.2% Phytagel and pH 5.8 (BAILEY et al., 1993). In the cotyledonary stage, these embryos were transferred to the medium MSM6 (activated-free charcoal MSM6AC) until the physiological maturity (FINER; McMULLEN, 1991) when they were desecated. The desecation process consisted of set up several embryos on Petri dishes which were

sealed with plastic film for two days. Thereafter, these embryos were transferred to the germination and conversion medium MSO composed of MS salts (MURASHIGE; SKOOG, 1962), B5 vitamin (GAMBORG et al., 1968), 3% sucrose, 0.2% Phytagel and pH 5.8. In this phase, the photoperiod was 23 hours to avoid premature flowering. As soon as the hypocotyl and radicle were visually detected, they were transferred to Magenta boxes (Sigma) with medium MSO for seedling elongation and development.

Acclimatization

Seedlings with well-developed radicular systems were transplanted into plastic containers filled with vermiculite and transferred into a growth chamber where they stayed for 20 days under the previous conditions of temperature and photoperiod. In this growth chamber they were protected by plastic film. Soon after, these seedlings were transplanted into plastic pots filled with soil for more 10 days. Thereafter, the plants were transferred to greenhouse conditions, and the photoperiod was reduced to 16h for seed production.

Experimental design

The experimental design was completely randomized with ten replications. Every experimental unit had ten cotyledons with their inner side on the growing medium. These data were reported by descriptive statistics.

Organogenesis

In this experiment, mature seeds were used as explants. First, the seeds were disinfested in 70% ethanol for 5 min., incubated in 2.5% sodium hypochloride (v v^{-1}) plus 0.01% Tween 20 for 20 min., rinsed four times in distilled, deionized and autoclaved water, and dried on sterilized filter papers. Next, these seeds were distributed on Petri dishes (90 x 15 mm) containing growing medium (GM) prepared with B5 salts, B5 vitamin (GAMBORG et al., 1968), 3% sucrose , 3 mM MES (2-N-morfolino-ethanosulfonic acid), 0.7% Phytagel and pH 5.8. Finally, the seeds were maintained at 27 ± 1°C, photoperiod of 16 hours, and irradiance of 24-36 μmol m^{-2} s^{-1} for 4 days.

Every cultivar had 50 normal seedlings collected from 80 germinated seeds. The explants were obtained by the method of Olhoft et al. (2003) after removing the seed tegument and excising the terminal end of hypocotyls below the cotyledonary node. Thus, two explants were obtained from every seed through the cotyledon separation along the remained hypocotyl. One hundred explants from every cultivar was set up on the induction medium (SIM) prepared with B5 salts (GAMBORG et al., 1968), B5 vitamin (GAMBORG et al., 1968), 3% sucrose, 3 mM MES, 1.67 mg L^{-1} BAP (6-benzilaminopurina), 0.6% Phytagel and pH 5.6. The Petri dishes were incubated under photoperiod of 16 hours and light irradiance of 24-36 μmol m^{-2} s^{-1} during two weeks for stimulating plantlets induction. Thereafter, the terminal end of the hypocotyl was eliminated, and the explants with newly developed plantlets were transferred to Petri dishes with fresh growing medium for more two weeks. Afterward, the cotyledons were removed and the plantlets transferred to the elongating medium (SEM). This medium consisted of MS salt (MURASHIGE; SKOOG, 1962), B5 vitamin (GAMBORG et al., 1968), 3% sucrose, 3 mM MES, 0.5 mg L^{-1} giberellic acid, 50 mg L^{-1} asparagine, 0.1 mg L^{-1} IAA, 1 mg L^{-1} zeatin, 0.7% Phytagel and pH 5.6. These media were subcultivated two times every 15 days. Individual plantlets with 3 cm in length were transferred into rooting medium (RM) with B5 salt (GAMBORG et al., 1968) at half strength, 3% sucrose, 3 mM MES, 1 mg L^{-1} AIB (indol-3-butíric acid), 0.7% Phytagel and pH 5.6.

Acclimatization

Seedlings with well-developed radicular systems were transferred to plastic containers filled with vermiculite, covered with plastic bags, and maintained in the growth chamber under the same conditions of temperature and photoperiod for 20 days. Next, they were transplanted to plastic containers filled with soil and grown for more 10 days. Thereafter, the plants were transferred to greenhouse conditions.

Experimental design

The experimental design was completely randomized with three replications of 100 explants per cultivar. Data from plantlets induction, number of plantlets elongating, number of elongated plantlets, rooting and plant regeneration were reported by their means.

Results and discussion

Embryogenesis

The present protocol permitted that somatic embryos could be differentiated in the inner surface of immature cotyledons during only four weeks (Figure 1). Concentration of 2,4-D at 40 mg L^{-1} has been efficient for inducing the development of somatic embryos in soybean cultivars (BUCHHEIM

et al., 1989). This efficiency, however, was highly discrepant among these cultivars. In six weeks and after two periods of sub-cultivations on the medium MSD40, the number of embryos per cotyledon ranged from 0.12 to 2.30 (Figure 2). The cultivar CD 201 developed an average of 2.3, CD 202 developed 0.98, CD 216 developed 0.86 and CD 220 developed 0.94 somatic embryos. Otherwise, CD 217 with 0.13 and CD 218 with 0.12 somatic embryos had small embryogenic capacity. Similarly, variability in the somatic embryogenesis of soybean genotypes was reported by Parrot et al. (1989), Komatsuda and Ko (1990) and Bonacin et al. (2000).

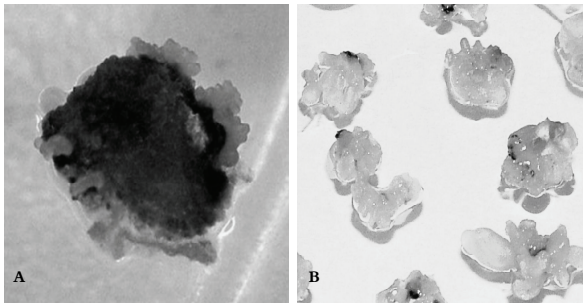

Figure 1. Clusters of somatic embryos from immature soybean cotyledons: A=Immature cotyledon cultivated for six weeks on MSD40 growing medium; B=Isolated somatic embryos on the MSD20 growing medium.

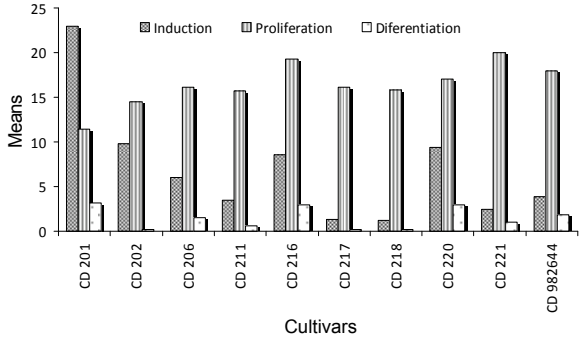

Figure 2. Average numbers of induction, proliferation and differentiation somatic embryos.

Every cultivar started the proliferation phase with different number of somatic embryos because all the globular embryos were inoculated on the medium MSD20. At the beginning of this phase, the somatic embryos developed from 0.87 to 2.0 globular embryos (Figure 3). In terms of similar number of inoculated embryos, the cultivar CD 216 developed 1.93 and the CD 221 developed 2.0 embryos. Although the CD 201 had the highest embryo production in the induction phase, it had the lowest number of embryos per inoculated embryo in the proliferation phase. These responses are similar to reports from Gesteira (2002) who found that the cultivar CD 201 was among the best cultivars in the induction phase, but the lower frequency in the proliferation phase. Weak and positive correlation between these phases has also been observed in some research reports (BAILEY et al., 1993, SIMMONDS; DONALDSON, 2000).

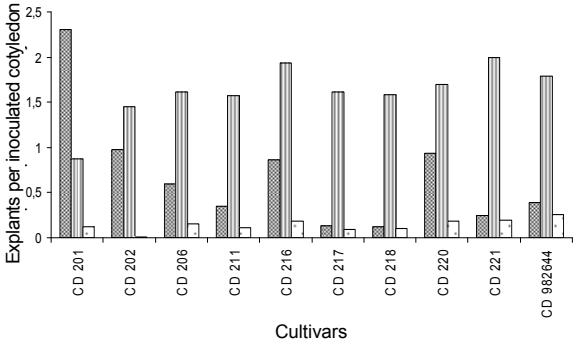

Figure 3. Number of embryos from inoculated embryos in several phases of somatic embryogenesis in soybean cultivars. Number of embryos per inoculated cotyledon; . Number of somatic globular embryos per inoculated embryo at the beginning of the proliferation; Differentiated embryos per inoculated embryo

In the present experiment, these soybean cultivars had marked influence on the induction of somatic embryogenesis as previously described through different protocols (BAILEY et al., 1993; KOMATSUDA; OHYAMA, 1988; KOMATSUDA et al., 1992; MEURER et al., 2001; RANCH et al., 1985; SANTARÉM et al., 1997; SHOEMAKER et al., 1991; SIMMONDS; DONALDSON, 2000).

In the differentiation phase, the growing medium without 2,4-D effectively inhibited the development of somatic embryos and allowed the differentiation of tissues. Morphological abnormalities were avoided by the excessive exposure of these embryos to the activated charcoal, therefore, reducing the residual effect of 2,4-D. Similar to auxins, other compounds from cultivated tissues have also inhibitory effects on the growth and development of these embryos (BUCHHEIM et al., 1989).

The somatic embryos obeyed the same pattern of development of the zygotic embryos transferred to the hystodifferentiation growing medium (Figure 4). After the globular stage, they differentiated into cordiforme, torpedo and cotyledonary stages. Evidently, the presence of abnormal embryos was detected during this stage.

The differentiation phase was less efficient than the induction and proliferation. Every somatic embryo produced from 0.014 to 0.25 differentiated embryos. The best genotype was the line CD 98-

2644, but the indices of differentiated explants from the cultivars CD 206, CD 216, CD 220 and CD 221 were above 10.0% unlike the CD 202, CD 211, CD 217 and CD 218 that had the lowest indices.

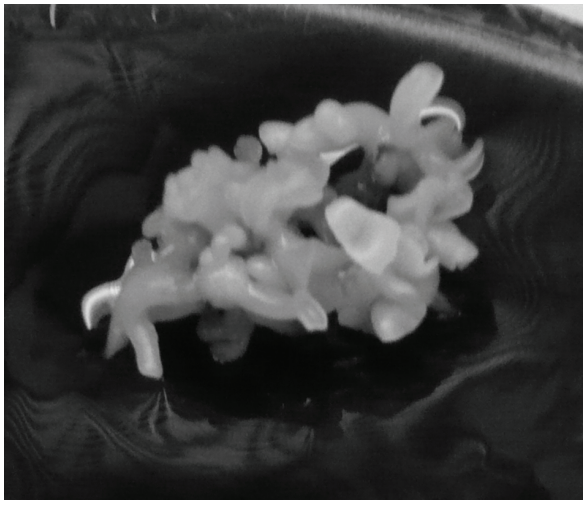

Figure 4. Somatic embryos of soybean cultivars from the end of hystodifferentiation phase growing on the medium MS6MAC.

Mature cotyledonary embryos were characterized by their color changes from green to creamy yellow, and time to physiological maturity was recorded between four and six weeks. Full maturation is unnecessary to obtain plants from somatic embryos, but it is required for reaching high levels of plant regeneration (BUCHHEIM et al., 1989). The regenerative potential from physiologically mature cotyledonary embryos that germinated on the medium MSO is showed in the Figure 5A. Desiccation is the best way to enhance the embryo germination which is generally low (LIU et al., 1994) although they are morphologically similar to the zygotic embryos (LIU et al., 1994).

Different capacity of regeneration was detected among the cultivars. The cultivar CD 201 with 82% had the best response, but the CD 220 had 58% and the CD 216 had the regenerative capacity of 51% which is a satisfactory level.

Loganathan (2010) studied the plant regeneration from somatic embryos of soybean and reported different responses from several media and acclimatization conditions under laboratory and greenhouse conditions. Although most of the embryos from the regulator-free growth medium developed the roots and shoots, some of them were incapable of overcoming the desiccation process. They showed some dark and necrotic appearance and did not germinate. The explanation is the long period of treatment with 2,4-D which may have affected the embryogenic potential.

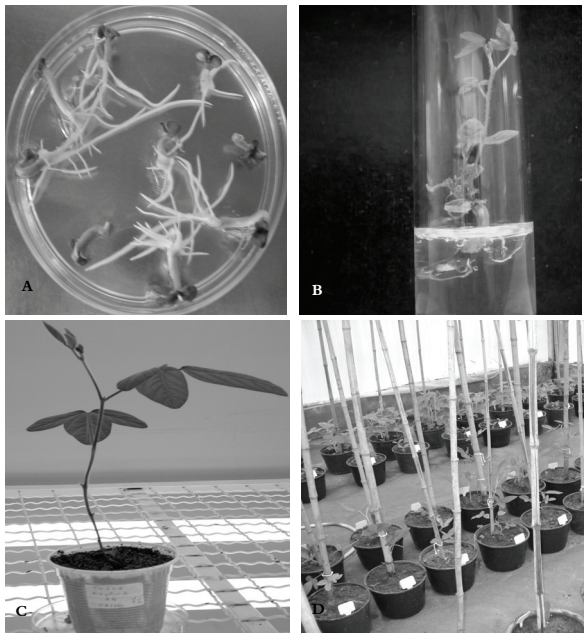

Figure 5. (A) Stems and well-developed radicles from the conversion and regeneration medium (MS0); (B) regenerated soybean seedling from the medium MSO; (C) acclimatization of regenerated plants *in vitro*; (D) plant acclimatization in the greenhouse.

In terms of total explants per immature embryo inoculated at the beginning of induction phase, the best responses were obtained from CD 201, CD 216 and CD 220, which are the preferential cultivars for somatic embryogenesis. The cultivar CD 201 highlighted in the present experiment because of its high capacity of induction, unlike CD 216 and CD 220 that had similar performance during the induction, proliferation, and differentiation phases. The best cultivar performance in some phase not necessarily is the best in another one because the variability depends on cultivar responses.

In soybean, some studies have demonstrated the effects of maturation groups on the induction of somatic embryogenesis. Shoemaker et al. (1991), Tian et al. (1994) and Tomlin et al. (2002) reported that soybean genotypes from early maturation groups had high embryogenic responses than cultivars from late maturation groups. In part, these responses are in agreement with the present results because the early cultivar CD 216 had similar performance in the induction, proliferation and differentiation phases. Furthermore, Ko et al. (2004) observed high embryogenic responses from genotypes classified in the intermediate maturation group. The present responses are also in agreement with these reports because the CD 201 and CD 220, which were efficient during the embryogenesis induction, are clustered in a semi-precocious group of maturation. Otherwise, Ranch et al. (1985) and

Bailey et al. (1993) did not find any relationship between maturation groups and somatic embryogenesis.

The present results can help plant breeders to select soybean cultivars and they show some alternatives that could be used to improve the efficiency of the somatic embryogenesis. An example is the protocol adjustment to permit the proliferation of CD 201 or induce the embryo development of the cultivars CD 216 and CD 220. Some cultivars as the CD 206 and CD 221 that differentiated few explants could be recommended for successful somatic embryogenesis if the induction could be increased. Others, as CD 211, CD 217 and CD 218 should be avoided because they were less efficient in two of three regeneration phases.

Organogenesis

All the cultivars had organogenic capacity of plant regeneration, and the plants developed in less than 90 days (Figure 6).

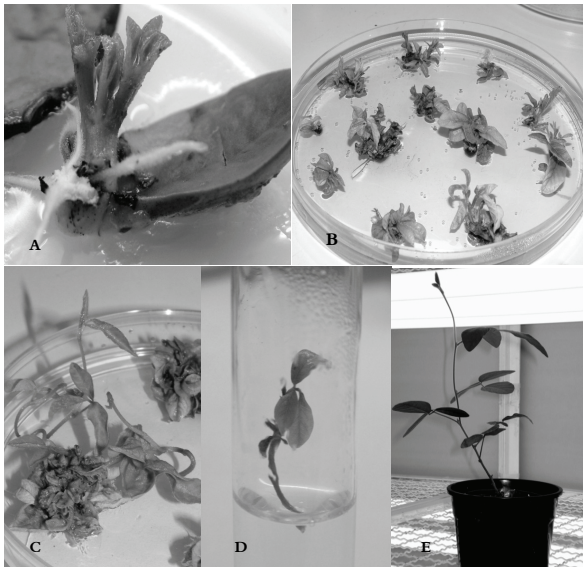

Figure 6. Organogenesis from soybean cotyledonary nodes: (A) Plantlets induction; (B) isolated plantlets from cotyledons in the beginning of the elongation phase; (C) elongated plantlets; (D) elongated plantlets and isolate in rooting medium; (E) acclimatization of regenerated plants.

From every cultivar, however, different responses were detected in the number of induced plantlets. The explanation for these findings may rest with differences in the seed quality of the explant sources or the cultivar performance *in vitro*. Similarly, these cultivars had different responses in number of elongate plantlets, number of seedling with roots and regenerated plants (Figure 7). The present cultivars had differences in the number of elongated shoots per plantlet, but the CD 217 was the most responsive (Figure 8).

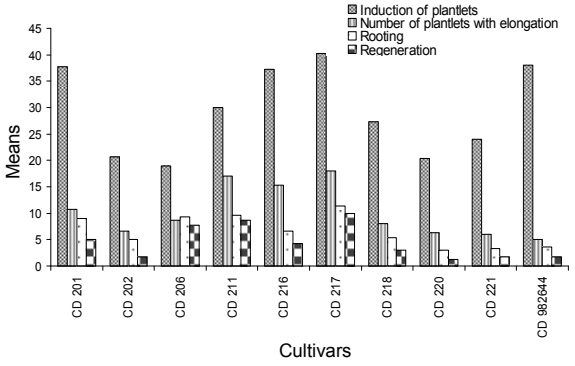

Figure 7. Number of differentiated structures during the organogenesis.

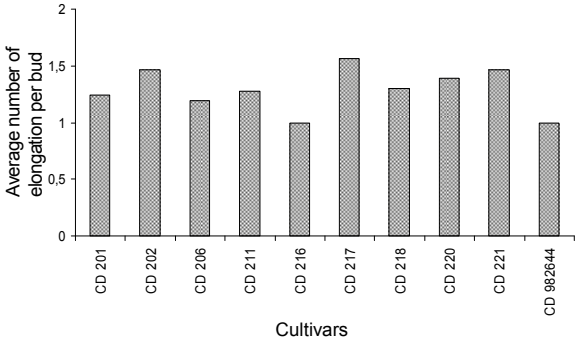

Figure 8. Number of elongations obtained by shoots that produced elongations

Elongated plantlets with the average length from 3 and 4 cm were isolated and transferred to test tubes with rooting media amended with 1 mg L^{-1} AIB where they remained for 15-20 days. These elongated plantlets were positively responsive to rooting. Again, the CD 217 was the best cultivar, and it was followed by those cultivars from intermediate maturation group as the CD 201, CD 202, CD 206, CD 218, CD 220 and CD 221.

As acclimatization of plants is a critical phase in tissue culture, only the plantlets with well-developed roots were acclimatized. Even so there were losses. For example, leaf necrosis followed by seedling death was observed between five and seven days after the transplanting into the vermiculite, but the cultivar CD 217 was still considered satisfactory.

Somatic embryogenesis and organogenesis periods

Cultivars with high frequency of embryogenesis or organogenesis must be selected to participate in genetic programs of plant transformation. However, some aspects must still be considered before choosing the regeneration pathway. One relevant

factor is time to plant development. In somatic embryogenesis, time to full development was ten months, and more 45 days was necessary to obtain the explants. Otherwise, the organogenesis is faster because time to completely developed soybean plants is only four months. Another factor is the level of plant regeneration, which was higher in plants submitted to organogenesis (88%) than in those submitted to embryogenesis (82%). In oat plants, Bered et al. (1998) reported the preferential regeneration through organogenesis.

Conclusion

The efficiency of somatic embryogenesis and organogenesis is affected by the plant genotypes. The regeneration of the soybean cultivars CD 201, CD 216 and CD 220 by somatic embryogenesis is more efficient. The regeneration of the cultivar CD 217 through organogenesis is more efficient. The embryogenic and organogenic systems can be used efficiently in the genetic transformation of soybean plants.

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