CHAPITRE VIII

PEARL MILLET REGENERATION FROM IMMATURE INFLORESCENCES: INFLUENCE OF EXPLANT AGE, *IN VITRO* GROWTH CONDITIONS AND PLANT GENOTYPE ON THE REGENERATION POTENTIAL

F. PINARD* et C. CHANDRAPALAIA
ICRISAT, Patancheru, 502324 A.P., Inde
* : Rhône Poulenc Agrochimie, Service Fongicide,
14-20, rue P. Baizet, 69009 Lyon

Ce travail présente une analyse du potentiel de régénération *in vitro* du mil à partir d'inflorescences immatures. Le comportement des explants y est décrit en termes de réponses embryogène et morphogène des cals induits (embryogenèse somatique), de caulogenèse et de rhizogenèse. Les mesures réalisées montrent le plus grand potentiel des inflorescences les plus jeunes dont la taille est inférieure à 2 cm, et la spécificité génotypique du phénomène de régénération, évaluée sur 10 génotypes différents de mils. Pour un cultivar, il est montré que l'apport d'ABA (0,1 mg.l^{1}) dans le milieu de culture favorise la réponse embryogène des explants.

I - INTRODUCTION

Pearl millet plants have been regenerated from various tissues, and the work on immature inflorescence is well documented (Vasil and Vasil 1982, Lu and Vasil 1982, Rangan and Vasil 1983, Botti and Vasil 1984, Swedlung and Vasil 1985, Ding Hou *et al* 1989, Talwar and Rashid 1990). As in the case of other cereals, it was demonstrated that regeneration occurs mainly through somatic embryogenesis.

Most authors agree on which nutrient salts support the best growth of explants (MS salt, Murashige and Skoog 1962). Also, there exists general agreement on the succession of plant hormones which has to be used to obtain regeneration (2,4 Dichlotophenoxyacetic acid (2,4 D), followed by Indole-3-acetic acid (IAA) or Naphtalene-acetic acid (NAA) mixed with Kinetin (K) or 6-Benzylaminopurine (BAP), then hormone free medium). However, the genotype influence of the mother plant on the explant regenerative potential is still being debated. Rao and Nitzsch (1984) and Mallikarjuna (1988) have published quantitative data showing the genotype specificity of pearl millet regeneration. Conversely, Vasil (1987) stated that if a genotype specificity exists, it is of minor importance as all of the pearl millet lines tested have shown regeneration potential. In order to further investigate the factors which influence pearl millet tissue culture, it is imperative to select a prototype that has the best regeneration potential. Therefore, the influence of genotype upon regeneration is of major importance and needs further clarification.

In order to evaluate the genotype specificity of the regeneration process, the work presented here aims to define standardised conditions for *in vitro* culture of pearl millet immature inflorescence. Shown is the influence of the inflorescence physiological stage on its regeneration potential and how the composition of the medium can influence this potential. Thus, following a standardized regeneration protocol, a comparison was made of regeneration potential between ten pearl millet lines .

II - MATERIAL AND METHODS

Pearl millet lines were greenhouse grown within a range of temperature from 20 °C to 40 °C. In all experiments, immature inflorescence were aseptically dissected by removing outer leaf sheaths after cleansing with 70° ethanol. Explants were cultivated in Petri dishes (diam. 5 cm or 10 cm) under artificial light (5000 lux, photoperiod 16h light/8h dark) at the temperature of 26 °C.

A - Developmental stage influence

Two lines were retained for this study, IPC 107 and 81B. To investigate the influence of the developmental stage of the inflorescence, the explants were collected at different times after floral induction, and cultivated successively on 3 semi-solid media, based on Murashige and Skoog (MS) basal salt: 20-30 days on CIM (Callus Induction Medium) = MS + 2.4 D 2 $mg.l^{-1}$, then subcultured every 20-30 days on RM (Regeneration Medium) = MS + K 0.5 $mg.l^{-1}$ + IAA 0.2 $mg.l^{-1}$; rooted plantlets were finally transferred on 1/2 MS = Half strength MS for establishment

B - Improvement callus induction and regeneration conditions

From lines IPC 107 and 81 B, inflorescence less than 2 cm long were harvested and prepared as described above. The procedure, summarised in Figure 1, compared 3 different culture conditions during callus induction each, in turn, followed by 3 distinct regeneration media

C - Comparison of 10 pearl millet lines regenerative potential

10 lines were selected: two inbreeds, IPC 107, IPC 94, and 8 lines from the a1 male sterile system: 842A/B, 81A/B, 841A/B and 863A/B. Respectively, 41, 37, 45, 47, 30, 27, 52, 58, 41 and 47 inflorescences were cultivated. They were prepared and cultured with the protocol described in the developmental stage experiment. From each lines, inflorescences less than 2 cm long were used.

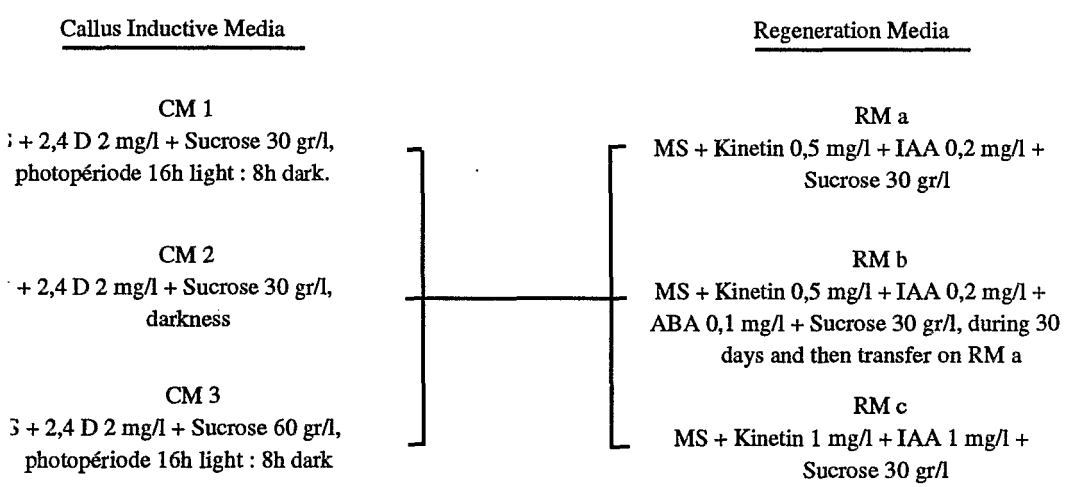

Figure 1
Improvement of callus induction and regeneration conditions of IPC 107 and 81 B

III - RESULTS

A - Influence of the inflorescence developmental stage

The developmental stage of the inflorescences can be followed according to the description given by Powers *et al.* (1980). On the smallest inflorescence (0,5 cm to 2 cm), the spikelets are rapidely proliferating, glume differentiation has just occured and floret primordia have just been initiated. On middle size inflorescence (2 cm to 4 cm), spikelets are well differentiate, stamen and pistil primordia are well initiated, palea start to elongate and a whorl of bristles are surrounding each spikelet. On the largest inflorescence (> 4 cm), hairs and bristles are enlarging, the inner glumes and palea are enclosing the flowers, stamens and carpel can be observed and the pedicelles start to elongate.

Figure 2 shows that IPC 107 has a good response for regeneration. It clearly appears that the younger inflorescence have the higher potential for regeneration: the number of calli obtained increases and the number of explants giving shoots (caulogenesis) is greater. The caulogenic process is faster for young tissues (40 days for the smallest inflorescence, 60 days for the inflorescence between 2 and 4 cm and 100 days for the biggest inflorescence) and younger tissues regenerate rooted plantlets more quickly. In the same way, it appears that the younger the inflorescence, the higher mean number of rooted plantlets obtained per explant (Figure 3; $\chi^2 = 12,6$, df = 2; P=O). Compared

with IPC 107, the efficency of regeneration for 81 B is lower (Figure 4): less calli are produced, very few plantlets are formed and the percentage of rooting is nearly nil. Yet, as with IPC 107, it is the youngest inflorescence that show the best regeneration potential.

B - Improvement of callus induction and regeneration conditions

Figure 5 shows the high potential for embryogenic callogenesis expressed by both lines. Influence of the different inductive conditions is minor and does not have significant effect.

1) Somatic embryogenesis and regeneration

a) IPC 107

- Influence of the callus induction conditions (CIM). Results obtained on IPC 107 vary with the CIM used (Figure 6a). After 60 days of culture, CIM 1 and CIM 3 led to some regenerative response which is not observed with CIM 2. At 90 days of culture, CIM 2 and CIM 3 induce a comparable number of regenerating plants ($X^2 = 4.87$, df = 4, P = 0.29) which remains low if compared to the number induced by CIM 1. But this effect is short lived and after 150 days of culture, no significant positive effect of any of the applied treatments is observed.
- Influence of the regeneration medium (RM). RMa significantly stimulates the regeneration potential from the inflorescence when applied after CIM 1 versus RMb or RMc. After 3 and 4 months of culture, the percentage of explant-producing plants is significantly higher with RMa than with RMb or RMc ($X^2 = 13.70$, df = 2, P = 0, T = 90 days). This effect is not noticed with either CIM 2 or CIM 3. None of the particular RM induces a significantly stimulating response. After 150 days of culture, all the treatments give a homogeneous result. Only RMb appears to have a negative effect on regeneration when its used is combined with CIM 3 ($X^2 = 24.4$, df = 8, P = 0).

b) 81B

Result obtained with 81B are more homogeneous than with IPC 107 (Figure 6b). No effect of CIM or RM is observed before 150 days of culture. At that time, the treatment CIM 2 followed by RMb induces significantly more regenerating explants than the others (35%), ($X^2 = 11.25$, df = 3, P = 0.01). This influence was noticeable after 120 days of culture and was similar to the one induced by RMa. But the RMa effect did not sustain and was reduced to nil after 150 days.

2) Comparison of 10 pearl millet lines regenerative potential

Figure 7 shows that rapidly, all the inflorescence produced embryogenic calli. After 90 days of culture, the mortality became important and the lines showed different regenerative patterns. 841 A/B, 842 A/B and 81 A/B expressed a homogeneous response giving 70% to 90% of explants producing embryogenic calli ($X^2 = 8.3$, df = 5, P = 0.15). Compared to this group of 6 lines, 863 B expressed a similar potential ($X^2 = 11.38$, df = 6, P = 0.09) but 863 A showed a higher response of nearly 100% of

callogenesis after 140 days of culture (X^2 = 17.43, df = 6, P = 0). IPC 107 and IPC 94 showed a similar callogenesis potential of about 30% after 140 days, significantly lower than the one expressed by 863 B (X^2 = 17.16, df = 2, P = 0).

As shown in Figure 8, the germination of somatic embryos on the two inbred lines is obtained earlier, after 60 days of culture, and on a higher number of explants (20 % to 25 %) after 90 days. Further development of the plants is not obtained due to the high rate of mortality (Figure 7). A similar pattern of development is observed on 842 A/B but delayed by 30 days if compared to the inbred lines. The number of regenerating explants of 842 A/B at 140 days of culture (15 % to 17 %) is similar to the amount observed on IPC 107 and IPC 94 after 90 days (X^2 = 0.53, df = 3, P = 0.8). 841A/B expressed the same kinetic of development as that of 842B, but the number of regenerating explants is lower. On 863A/B and 81A/B, embryos started to germinate only after 90 days of culture. The number of regenerating explants from 863A is similar to the one of 842A/B (X^2 = 0.17, df = 2, P = 1) while 863B expressed as many regenerating explants as 841 A/B lines. Compared to 841A, 81A/B lines shows the smallest number of regenerating explants (X^2=0.94, df=2, P=0.61).

IV - DISCUSSION

The results we obtained show that the media and the chronology of subculturing needs to be defined in order to obtain a high level of regeneration. Under the most optimal conditions, the highest level of regeneration (50/133 = 38 %) was obtained on the young inflorescence (< 2 cm) of IPC 107. The developmental stage of an inflorescence appears to be of great importance as it controls the regenerative potential of the explants. The youngest inflorescence showing the highest capability of regeneration under the tested conditions.

The good regenerative potential of IPC 107 is slightly affected by the various growing conditions tested. When 2,4 D 2 $mg.l^{-1}$ is used for callus induction and IAA plus Kinetin for regeneration, the process of differentiation is more rapid. Neither alterations of luminescence and sugar content of the CIM nor alterations in hormone content of the RM increased the regenerative response.

Compared to IPC 107, 81B appears to be a cultivar with a lower potential for regeneration. None of the treatments used gave a result as good as those obtained with IPC 107. The best result with 81B was obtained under dark conditions and 2,4 D for callus induction and a 30 days exposure to ABA followed by kinetin plus IAA treatment to stimulate somatic embryogenesis. In this case, the response of 81B was better than the one obtained in previous experiments when the treatments corresponding to 2,4 D 2 $mg.l^{-1}$ and normal photoperiod (16 h / 8 h) were used to induce callogenesis followed by Kinetin plus IAA for induction of somatic embryogenesis .

The results of 81 B show that it may be possible to improve the regeneration potential by applying ABA. ABA, when applied to the inflorescence of 81 B, enhanced somatic embryogenesis. This agrees with the previous report of Rajasekaran *et al.* (1987) who found ABA was an important factor controlling the embryogenic capacity

of leaf explants from Napier grass (*P. purpureum*). Our finding demonstrates that ABA influence is genotype specific: ABA affects 81B more than IPC 107. With the use of ABA, an interesting level of regeneration is reached which should allow the development of studies and applications with 81B.

When compared under the same growing conditions, the 10 pearl millet lines tested each show a distinct regenerative response which makes possible to classify them into different groups. IPC 107 and IPC 94 display the best characteristics for regeneration. Embryogenic callogenesis and somatic embryogenesis occur rapidly from several explants. Unfortunately, the mortality expressed by the two lines limits the number of regenerating inflorescence and the final number of plants obtained is low (Fig. 8). With IPC 107, this result was not expected and conflicts with the result of the previous experiments. No explanation can actually be given to this phenomenon nor why only the two inbred lines are concerned.

Lines like 842A/B and 863A appear to have the same interesting potential for regeneration but delayed by 30 or 60 days when compared to IPC 107 or IPC 94. The calli obtained from these lines are still growing and further differentiation of plants can be expected. This observation shows that time can be also an important parameter which control the regeneration process and should be taken into consideration for a correct assessment of regeneration potential.

Line 863B gives less regenerating explants than 863A. Such difference appears to be linked to the male sterile system a1, but it is not clear why it affects only 863 and not the other lines of the same male sterile system used in this trial.

V - CONCLUSION

As mention for other cereals (Sun and Chu, 1986), immature inflorescence of pearl millet have proven to be a good material for *in vitro* regeneration. High yields of calli which regenerate plantlets can be obtain.

We demonstrate that the smallest inflorescence express the highest capability of regeneration under the tested conditions. This result agrees with the result of Botti and Vasil (1984) who obtained greater somatic embryogenesis with smaller inflorescence. More generally, it fits with reports from inflorescence on rice (Shi *et al.*, 1985), barley (Krumbiegel-Schroeren *et al.*, 1984) and Sorghum (*S. arundinaceum*, Boyes and Vasil, 1984) but conflicts with reports on maize (Suprasama *et al.*, 1986), orchard grass (Conger and Mac Donnell, 1983) or *S. bicolor* (Cai and Buttler, 1990) which show either that well-developed inflorescence produced more shoots or that developmental stages do not critically affect regeneration process. These observations suggest that the influence of the developmental stage on immature inflorescence regenerative potential is genus or species specific.

Our results show that genotype specificity exists for the regeneration process of pearl millet. Thus, they support the views of Rao and Nitzsch (1984) and Mallikarjuna. This, however, is in conflict with the assessment of Vasil (1987). It seems that Vasil discussed only the qualitative aspect of regeneration and did not consider the possibility

of low or high rates of regeneration. More generally, our findings agree with results from other cereal species which demonstrate that regeneration is genotype dependant (Duncan *et al.*, 1985, Bapat *et al.* 1988).

Regenerative potential can be described in terms of percentage of regeneration and in regeneration kinetics. These two parameters were shown to be very useful for the characterization of lines and will help to choose the most suitable ones for *in vitro* developmental studies and biotechnology applications. For example, line showing fast regeneration kinetic may be interesting to prevent from mutant regeneration (somaclonal variability) when not desirable.

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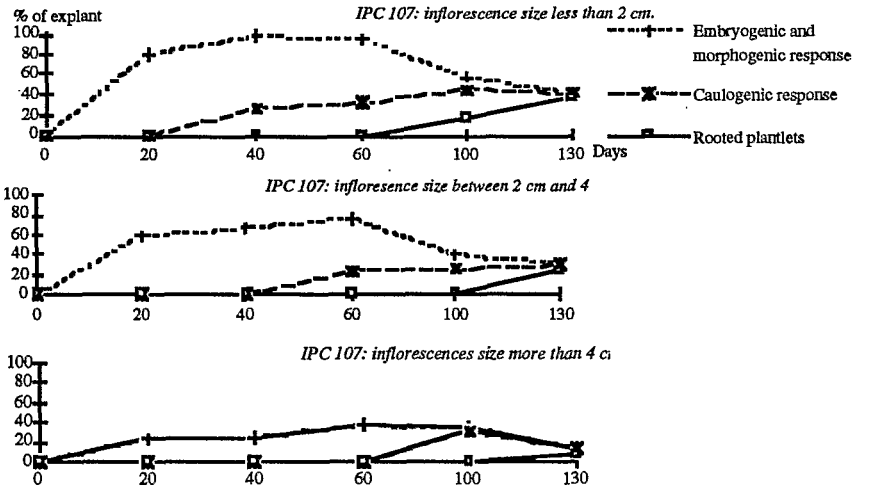

Figure 2

IPC 107- Influence of immature inflorescence developmental stage on their regenerative potential.

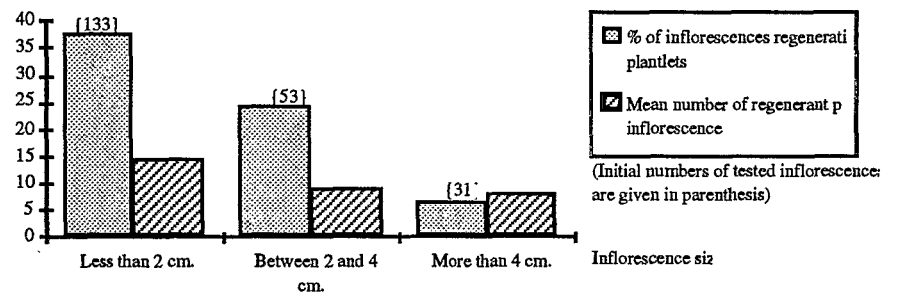

Figure 3

IPC 107- Influence of immature inflorescence developmental stage on the average yield of plant regeneration.

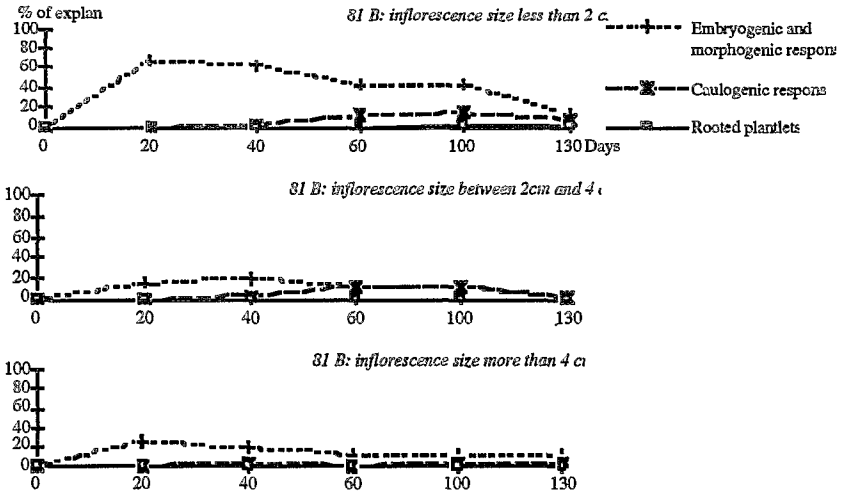

Figure 4

81 B- Influence of immature inflorescence developmental stage on their regenerative potential.

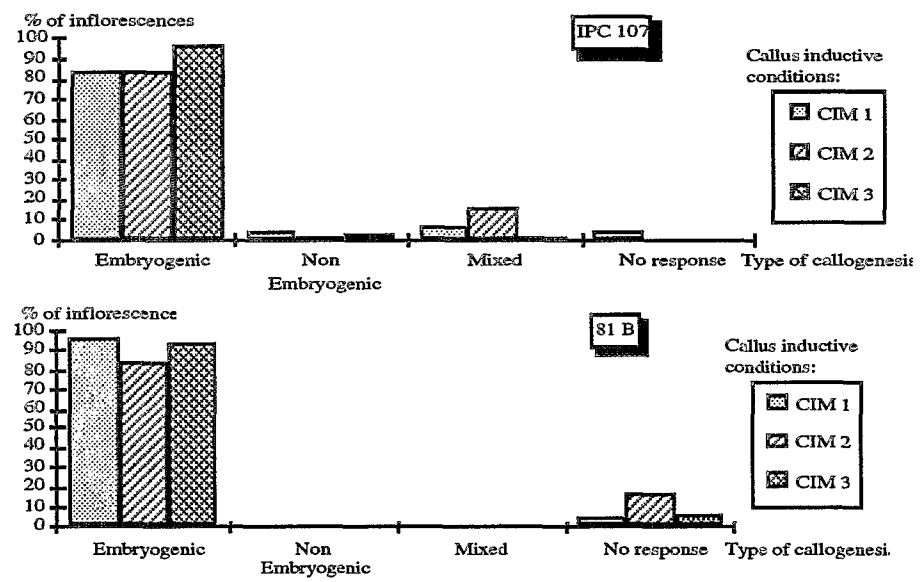

Figure 5

Callogenic response from IPC 107 and 81B immature inflorescences cultivated on 3 Callus Inductive Media (CIM 1,2,3)

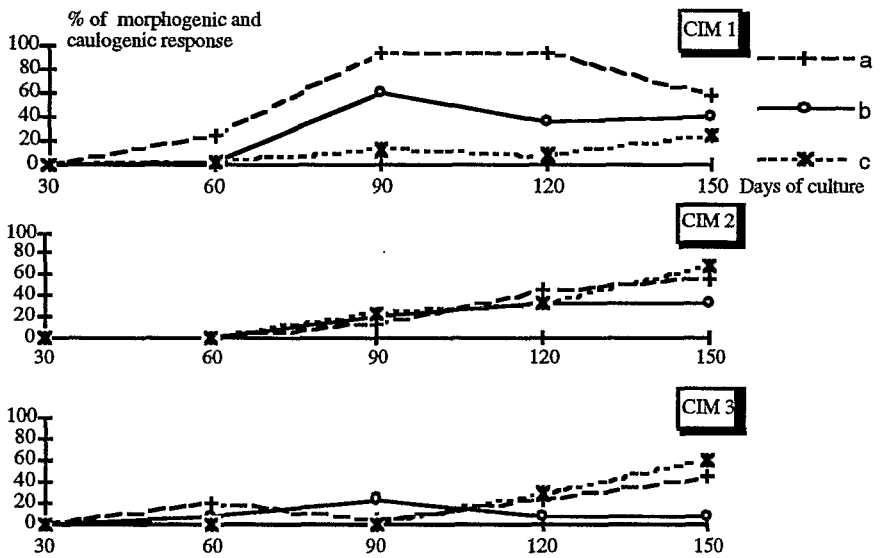

Figure 6a

IPC 107- Influence of 3 Regenerative Media (a, b, c) on the development of explants cultivated 30 days on 3 Callus Inductive Media (CIM 1, 2, 3)

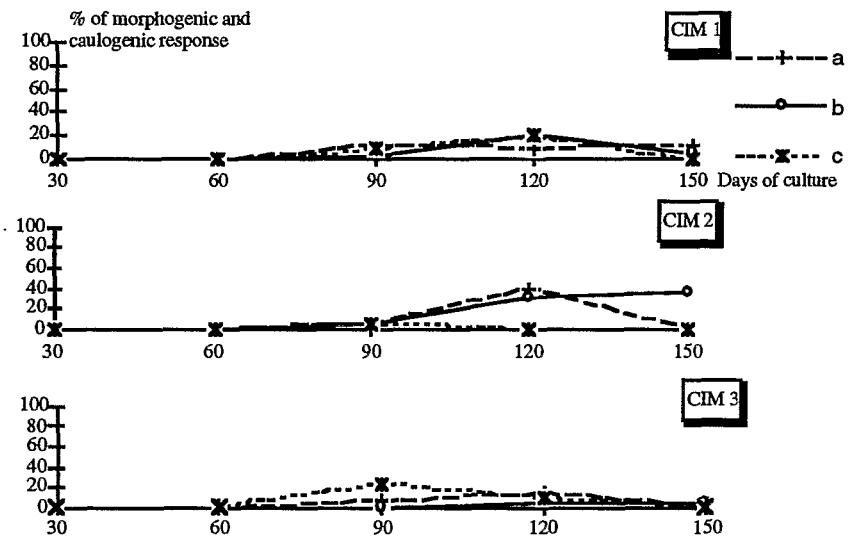

Fig. 6b

81 B- Influence of 3 Regenerative Media (a, b, c) on the development of explants cultivated 30 days on 3 Callus Inductive Media (CIM 1, 2, 3)

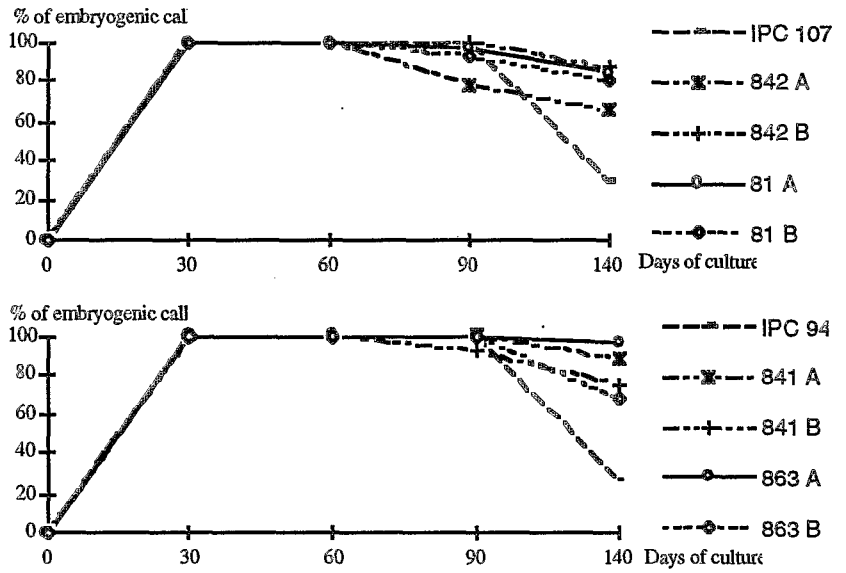

Figure 7
Somatic embryogenesis of pearl millet line IPC 107, IPC 94, 842 A/B, 81 A/B, 841 A/B and 863 A/B.

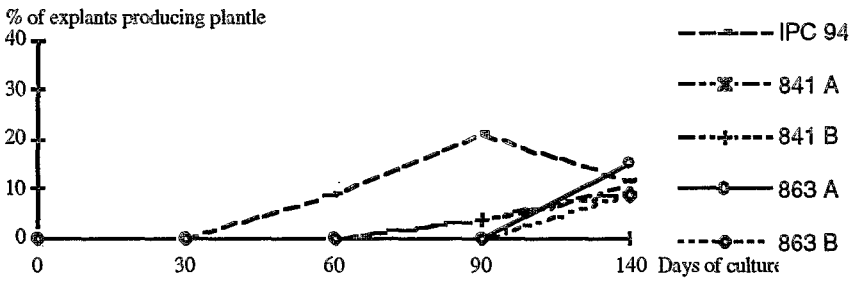

Figure 8
Plantlet regeneration from immature inflorescences of pearl millet lines IPC 107, IPC 94, 842 A/B, 81 A/B, 841 A/B and 863 A/B.