# CHAPTER FOUR
# RESULTS AND DISCUSSION

### 4.1. Callus induction of Maize

The immature embryos of SD7 and SD34 were used those length was 1.6-2.0 mm. Immature embryo was selected from ears of pure line. The embryos were selected and cultivated in two different culture medium (D and N6E). After three times of cultivation two types of callus were produced (see Figure 8).

Every 21 d when cultured *in vitro* can originate Type I or Type II embryo genic calluses, Type II was selected and transferred to fresh medium (Figure 8 - stage 4). The results indicated clearly that, Type I callus is formed by hard, compact and yellowish tissue, usually unable to regenerate plants, on the other hand Type II callus is soft, friable, highly embryo genic and able to regenerate a higher number of plants.

Results showed that, Type II embryo genic callus can regenerate only. These results not in aline with **Frame *et al.* (2006)**, who found that both types of calluses can be used to generate for tissue culture. The present results are in accordance with those reported by **Vassil *et al.* (1991)** and **Armstrong and Green, (1985)** who found that Type I is compact, white that failed to regenerate and Type II is friable, yellowish and able to regenerate.

Data in Figure 6 and 7 showed the overall of alive and dead callus for the selected varieties SD7 and 34 in respect. The highest values for alive callus were 260 and 180 in SD7 and 34, respectively.

While the lowest values were 137 and 120. The overall of alive callus was 190 and 148 and dead callus was 76 and 2.17, in respect. Although varieties were inbred lines, SD34 was more efficient than SD7 and the percentage of dead callus was 2% compared with the other variety was 40% (Figure 6 & 7)

Efficient shoot regeneration is vital for the establishment of a maize tissue culture system. Partial desiccation has been demonstrated as a tool for promoting embryogenesis and plant regeneration in both wheat (Carman 1988), and in indica rice (**Tsukahara and Hirosawa 1992; Rancé *et al.* 1994; Jain *et al.* 1996**)

Maize plant regeneration can take place through two avenues, that is, organogenesis or somatic embryogenesis. Somatic embryogenesis is the most common avenue of plant regeneration **(Odour *et al.* 2006).** With the rapid

development of tissue culture techniques, many types of explants, including gametic embryo and leaf tissue had been successfully regenerated into plants by tissue culture **(Aulinger *et al.* 2003, Huang and Wei 2004 and Ahamadabadi *et al.* 2007).** But at present, the most popular is still immature zygotic embryo in maize transformation, owing to simple inoculation operation and facile callus induction **(Binott *et al.* 2008).**

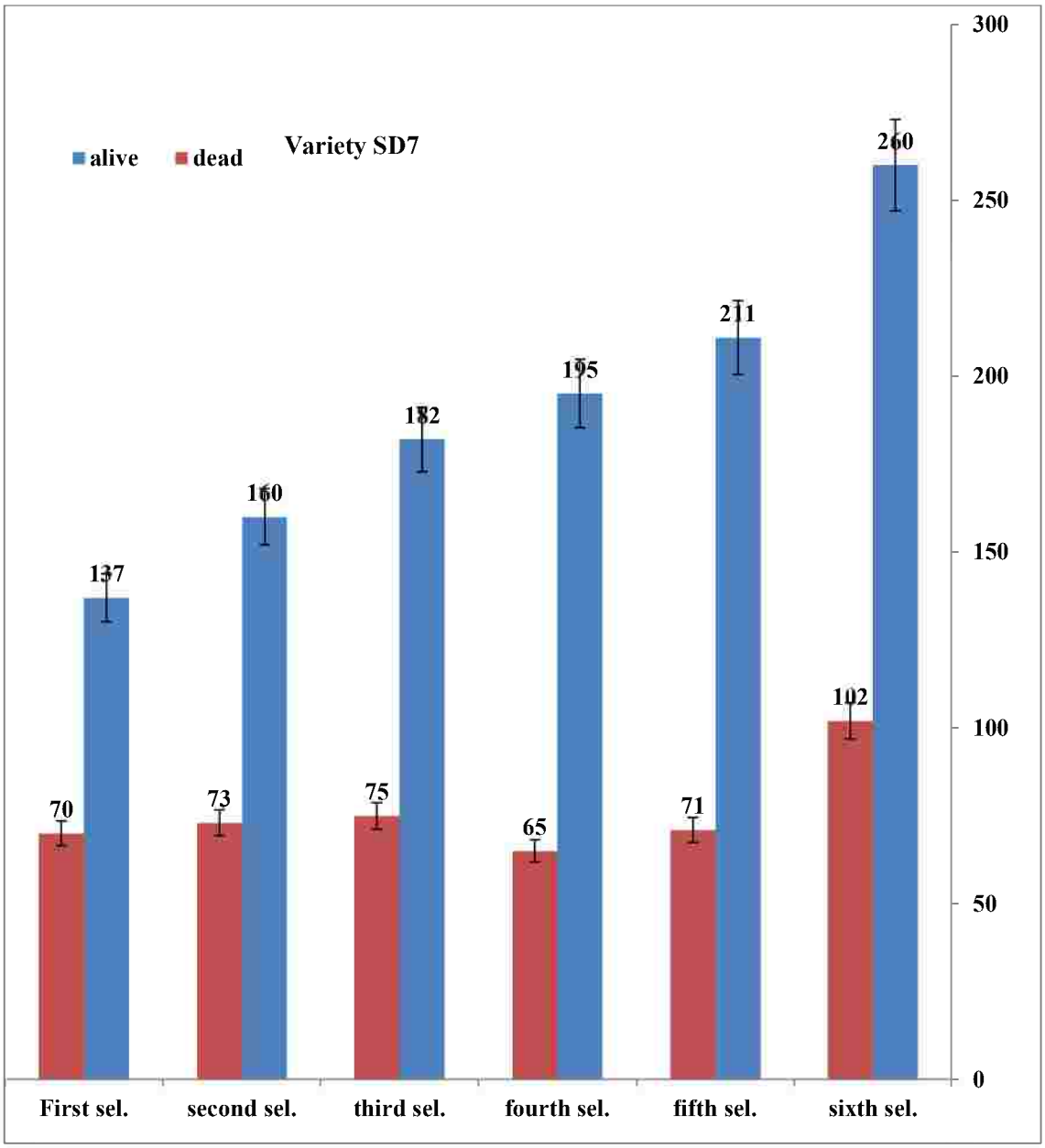


**Figure (6). The number of alive and dead calluses after six selections on N6E medium for variety SD7**

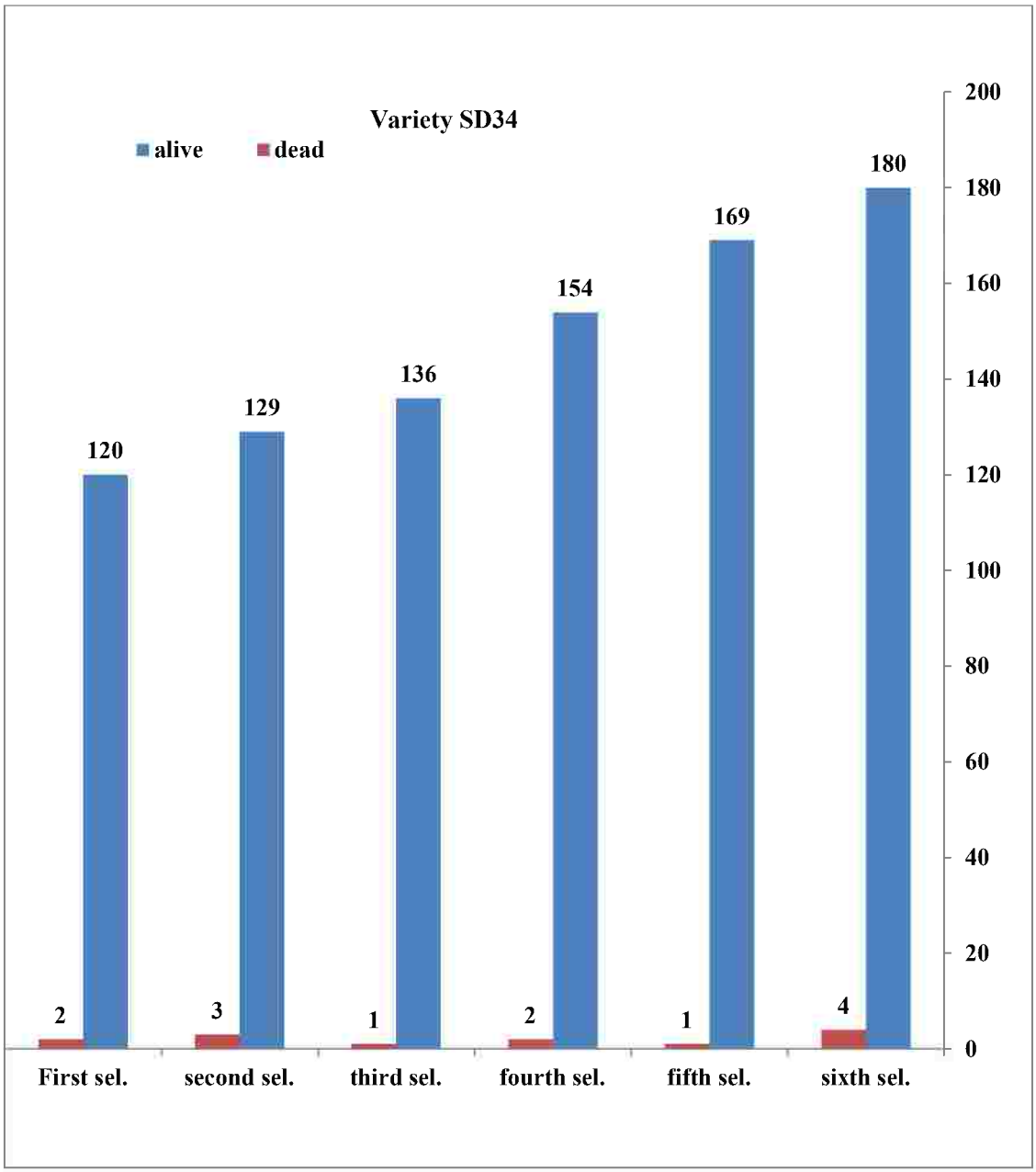


**Figure (7). The number of alive and dead calluses after six selections on N6E medium for variety SD34**

In the present results we have demonstrated that tissue culture technique can result in a significant and practical increase in callus production during the early phase of induction, which, from a practical perspective, can speed up the shoot regeneration course in maize. This simple and inexpensive technique is now available as a component for the efficient increasing of maize plants.

Every 40 days for three times we calculated the dead and alive callus as shown in Table xx. Almost 40% after 40 days of the callus were dead in SD7 and on the other hand in SD34 were nearly 80% of the callus were alive. The maximum number of alive callus were 28in SD7 forwared by 23 for SD34.

For the second read (80 days) results in Table xx showed the alive callus increasing while the dead callus were deceasing. For example the highest number was± 11.44in SD34 compared with± 14.44in SD34 (Table xx). Finally, after 120 days of the callus cultured the results achieved the same trend, although both dead and alive callus increasing. The overall of alive callus after the 120 days were ±10.44 in SD34 compared with± 13.99in SD7. (Table xx)

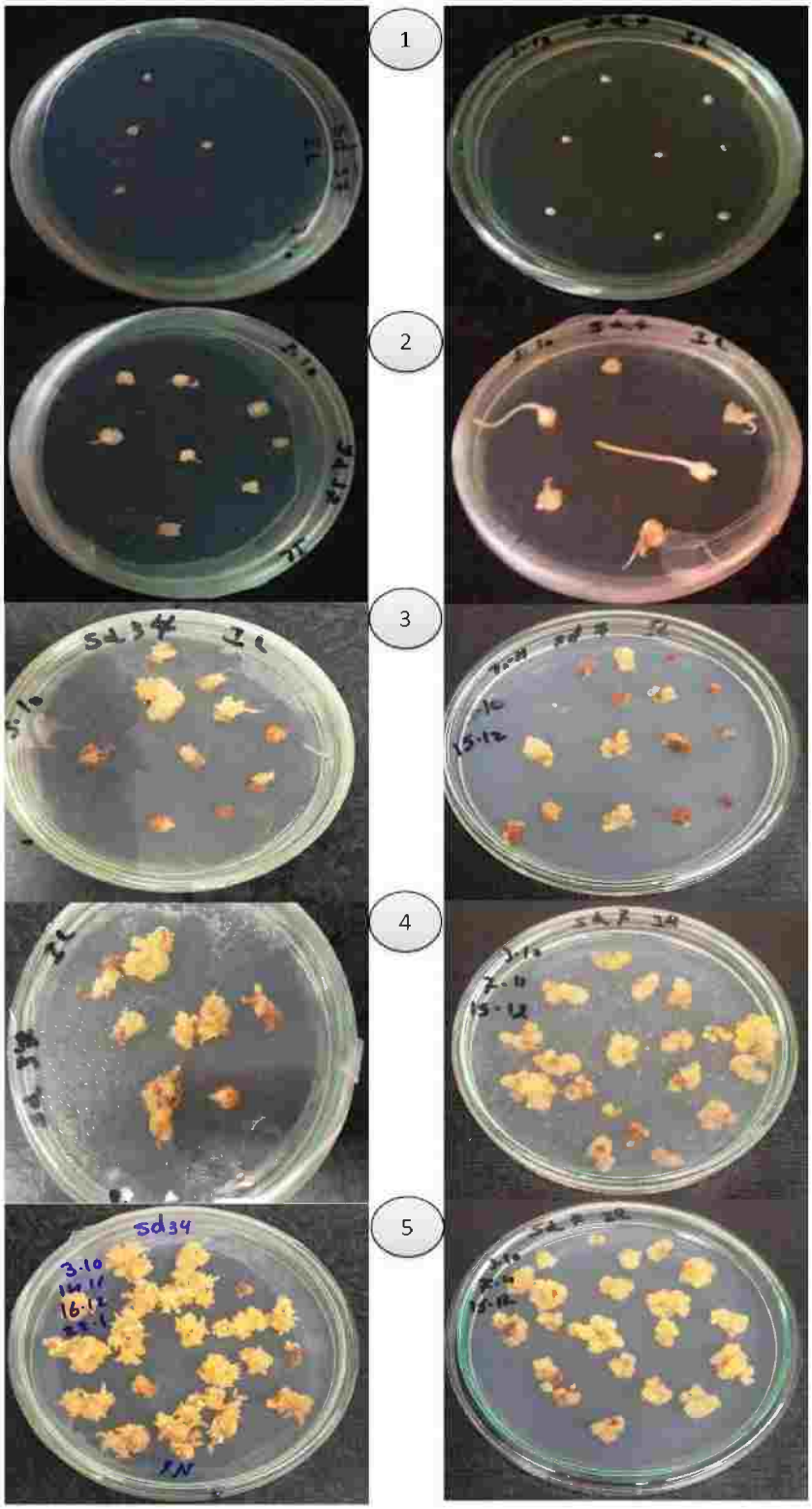


**Figure (8). The different growth stages for embryo cultured on medium D and N6E on variety SD7 (Right) and SD34 (Left) as follow: (1) planting of immature embryo; (2) embryo growth and forming; (3); Alive and dead callus (4) callus induction and (5) sub-culture of callus.**

**Table (1). Dead and a live callus of two inbred maize line used in the current resarsch**

| Inbreed lines | | SD7 | | SD34 | |
|---|---|---|---|---|---|
| Reading | | Dead | A live | Dead | A live |
| Read 1 (40 days) | Average | 6.42±1.2 | 14.54±1.4 | 3.14±0.30 | 11.78±1.77 |
| | Maximum | 23.00 | 28.00 | 15.00 | 23.00 |
| | Minimum | 0.00 | 5.00 | 0.00 | 0.00 |
| Read 2 (80 days) | Average | 5.04±0.47 | 14.44±1.55 | 2.64±0.23 | 11.44±1.23 |
| | Maximum | 23.00 | 26.00 | 12.00 | 24.00 |
| | Minimum | 0.00 | 0.00 | 0.00 | 0.00 |
| Read 3 (120 days) | Average | 3.74±0.36 | 13.04±1.33 | 3.88±0.11 | 8.10±0.89 |
| | Maximum | 15.00 | 27.00 | 9.00 | 20.00 |
| | Minimum | 0.00 | 0.00 | 0.00 | 0.00 |
| **Overall** | | **5.06** | **13.99** | **3.22** | **10.44** |
| **Total** | | **19.05** | | **13.66** | |
| **Percentage** | | **26.56** | **73.43** | **23.57** | **76.42** |

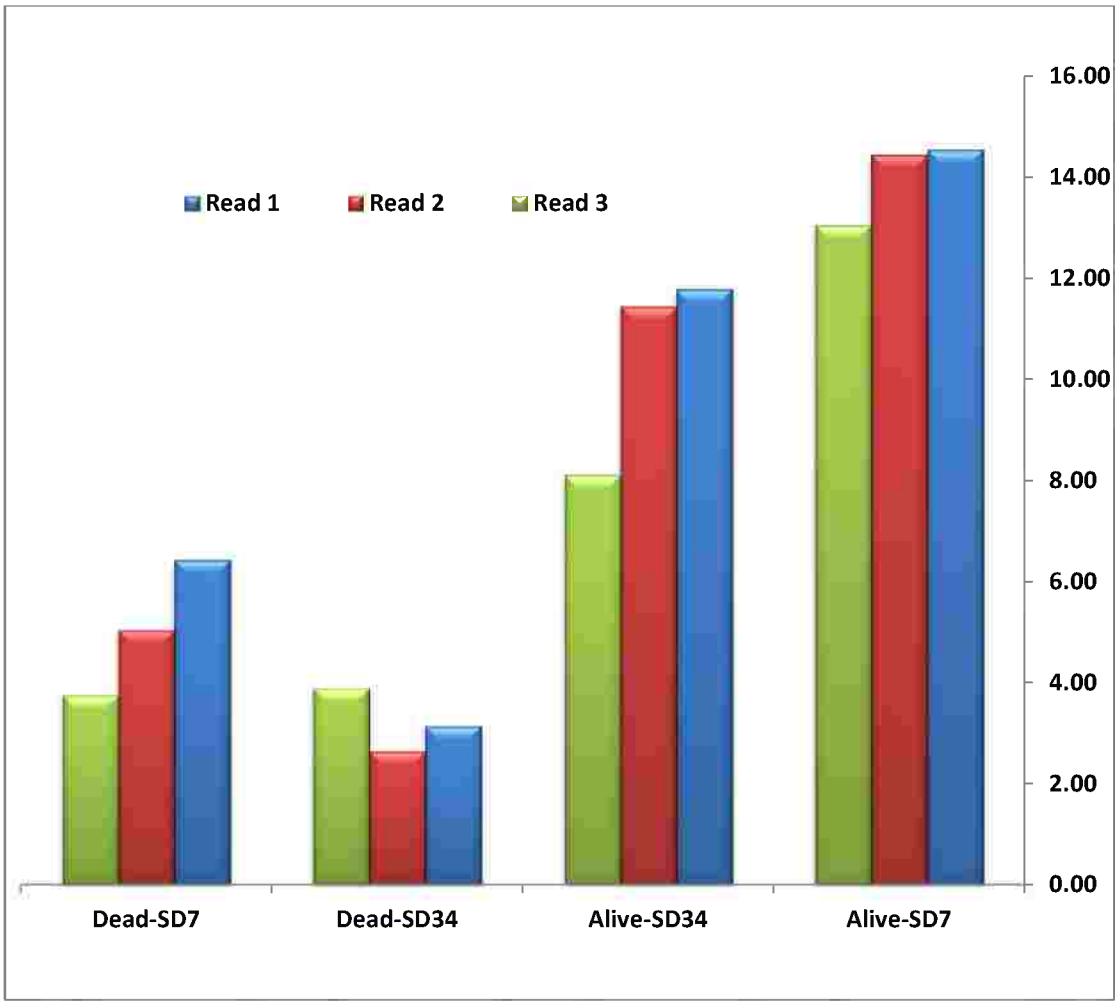


**Figure (9) Different values of dead and a live callus during the three reading**

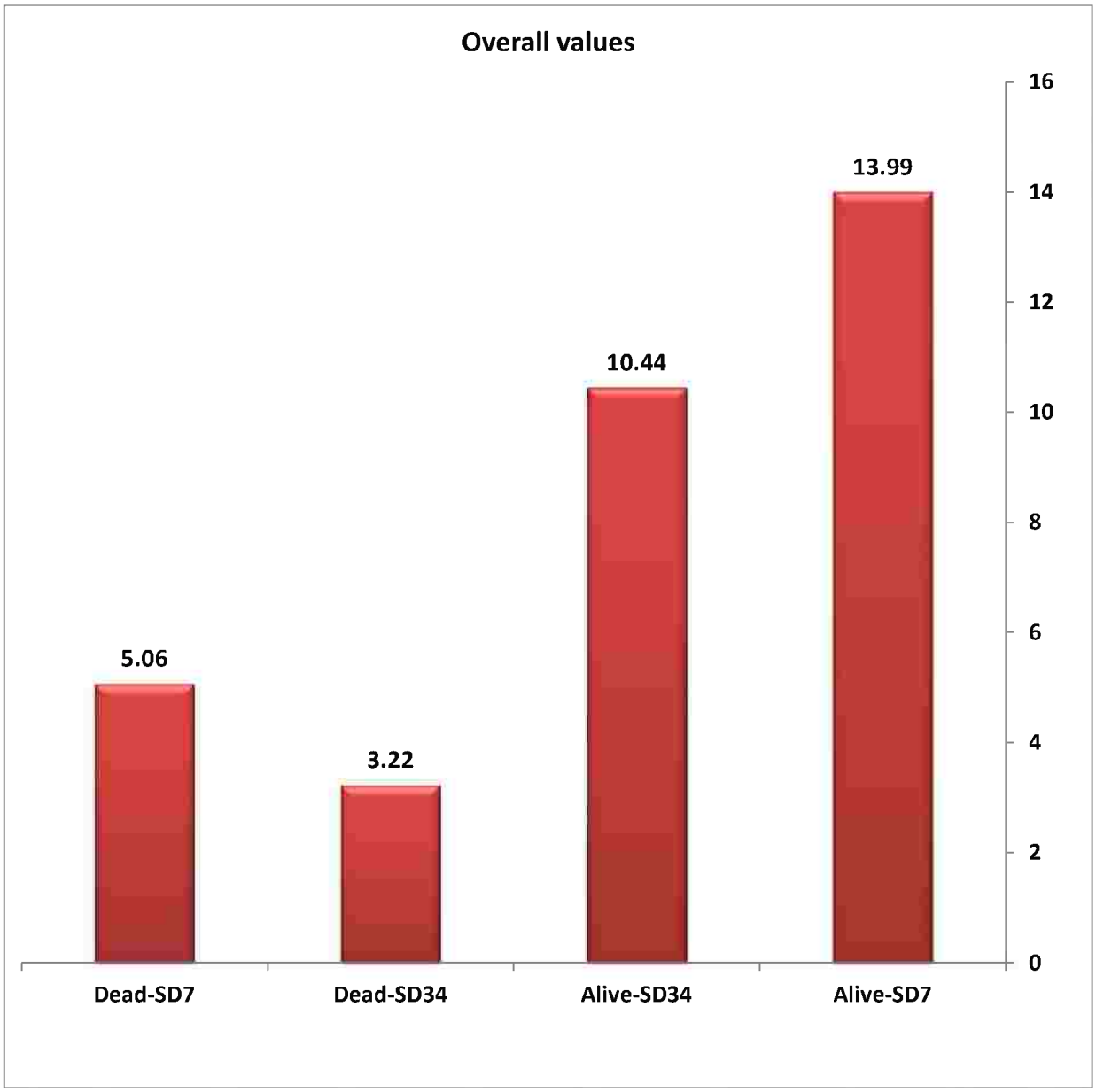


**Figure (10). Overall values of dead and a live callus for SD7 and SD 34 inbreed lines**

### 4.2. Selection of different maize genotypes:

Resistant calluses from the lines began to differentiate after 2 to 3 weeks, compared with non-transgenic lines. In order to find out the optimal parameters for the bombardment transformation of maize the expression plasmid containing bar gene as selectable marker and COMT anti-sense which express O-methyltransferase.

Three concentrations of PPT inhibitor (3, 4 & 5 mg/l) were used on selection medium to get transgenic lines. Transformation frequency for 3 mg/l PPT (59.3 and 60.2 %) for 900 and 1100 Psi and (33.9, 34.3, 35.2 and 26.5) for 4 and 5 mg/l PPT, respectively, shown in Table (3-5) and Figure (11).

Fifty plants came from two concentrations of PPT COMT-Anti sense detected by PCR analysis using selectable marker bar primers to get 311 bp as shown in Figure (11).

Figure (11) suggests that a single integration site for the COMT-antisense gene occurred in the genome of the transgenic plants. Each of the transgenic lines showed a unique hybridization band, which indicated that the pZMAS COMT (10.907 bp) was integrated into the genome of the host plants. No hybridization band was detected from genomic DNA of wild type plant

Maize inbred lines SD7 and SD34 were used in the current experiment. Maize is a cereal crop with superiority inbred lines and a complex genetic background. Calluses induced from inbred lines were recovered from N6 culture medium. Two types of calluses produced type I soft, hard and, unable to regenerate and type II, which used in this study friable, yellish and capable to regenerate.

The present experiments suggested that for some genotypes 2 mg/l of 2,4-D was the most suitable concentration for induction of embryogenic friable callus, which agrees with the report of (Bronsema et al., 2001).

Particle bombardment is still the method of choice for multiple gene co-transformation because the procedure is highly efficient particularly for monocot species and is convenient to use for large numbers of samples. (Hadi et al., 1996; Chen et al., 1998)

Our finding was that lignin content was as highly variable within a transgenic family as between transgenic families compared with control. Considerable somatic instability of transgene expression was observed when we

sampled 50 transgenic plants, that previously had reduced O-methyltransferase activity.

However, most of the studies of transgenic tobacco and poplar with down regulated COMT activity have shown that the lignin ratio was significantly reduced, but the lignin level of the transgenic plants was similar to the control even when the COMT activity was reduced (Atanassova et al., 1995; Vailhe et al., 1996; Lapierre et al., 1999).

Contrary to those reports, Sewalt et al. (1997) reported a moderate decrease of lignin content in their COMT down regulated transgenic tobacco plants. More recently, Guo et al. (2001) reported that down regulation of caffeic acid 3-O methyltransferase (COMT) reduced lignintransgenic alfalfa (Medicago sativa L.).

Down regulation of COMT modifies cell wall composition and enhances digestibility in field-grown maize. It is well documented that transgene expression varies among independent transformation events, individual plants from the same transformation event, and progeny of a transgenic plant (Spencer et al., 1992; Walter et al., 1992; Zhang et al., 1996).

All the genetic backgrounds enhanced digestibility as a result of introduction of the COMT transgenic. The extent of the effect of the COMT transgenic was dependant on the genetic background, especially for lignin content and cell wall digestibility. The COMT transgenic in some genetic backgrounds enhanced digestibility reaching similar levels those observed in the previous studies. This research has shown that genetic engineering using antisense technology can be used to down regulate COMT activity, which in turn can reduce lignin biosynthesis and modify the lignin molecule

**Table (2). Number of calluses living and dead on selection medium N6S + PPT 3 mg/L**

| PPT 3 mg/L | | | |
|---|---|---|---|
| **Number of calli** | **a live** | **dead** | **Number of live calli** |
| **double 900** | | | |
| 28 | 14 | 14 | 50.0 |
| 26 | 18 | 8 | 69.2 |
| **54** | **32** | **22** | **59.3** |
| **double 1100** | | | |
| 41 | 28 | 13 | 68.3 |
| 27 | 12 | 15 | 44.4 |
| 31 | 24 | 7 | 77.4 |
| 29 | 13 | 16 | 44.8 |
| **128** | **77** | **51** | **60.2** |
| **Control** | | | |
| 33 | 7 | 26 | 21.2 |

**Table (3). Number of calluses living and dead on selection medium N6S + PPT 4 mg/L**

| PPT 4 mg /L | | | |
|---|---|---|---|
| **Number of calli** | **a live** | **dead** | **Number of live calli** |
| **double 900** | | | |
| 35 | 12 | 23 | 34.3 |
| 27 | 14 | 13 | 51.9 |
| 33 | 9 | 24 | 27.3 |
| 30 | 8 | 22 | 26.7 |
| 25 | 4 | 21 | 16.0 |
| 30 | 14 | 16 | 46.7 |
| 180 | 61 | 119 | 33.9 |
| **double 1100** | | | |
| 33 | 16 | 17 | 48.5 |
| 28 | 8 | 20 | 28.6 |
| 27 | 7 | 20 | 25.9 |
| **88** | **31** | **57** | **35.2** |
| **Control** | | | |
| 39 | 13 | 26 | 33.3 |

**Table (4).Number of calluses living and dead on selection medium N6S + PPT 5 mg/L**

| PPT 5 mg/L | | | |
|---|---|---|---|
| **Number of calli** | **a live** | **dead** | **Number of live calli** |
| **double 900** | | | |
| 28 | 8 | 20 | 28.6 |
| 26 | 6 | 20 | 23.1 |
| 26 | 8 | 18 | 30.8 |
| 24 | 11 | 13 | 45.8 |
| 24 | 10 | 14 | 41.7 |
| 28 | 10 | 18 | 35.7 |
| 28 | 12 | 16 | 42.9 |
| 29 | 8 | 21 | 27.6 |
| **213** | **73** | **140** | **34.3** |
| **double 1100** | | | |
| 28 | 9 | 19 | 32.1 |
| 25 | 7 | 18 | 28.0 |
| 41 | 12 | 29 | 29.3 |
| 41 | 8 | 33 | 19.5 |
| 27 | 7 | 20 | 25.9 |
| 162 | 43 | 119 | 26.5 |
| **Control** | | | |
| 35 | 13 | 22 | 37.1 |

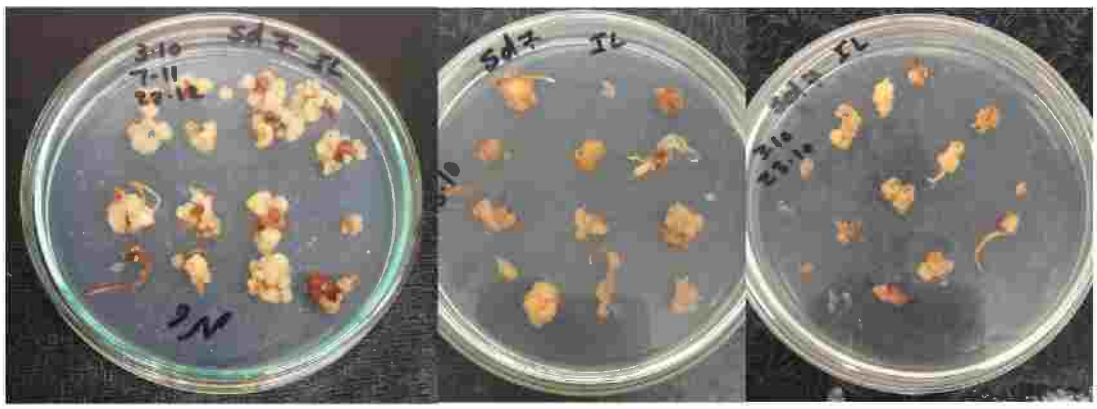

**Figure (10) Morphology of different types of callus from left to right 5, 4 and 3 mg/L PPT after bombardment**

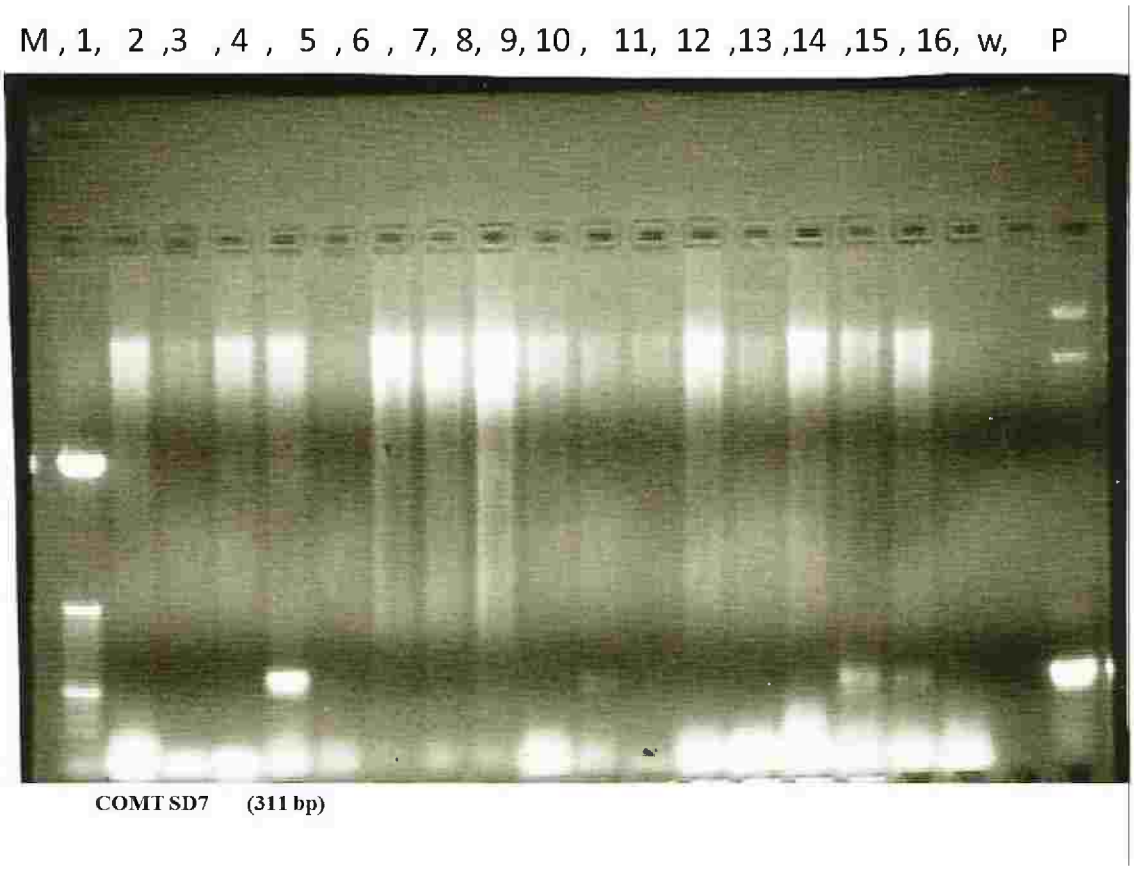


**Figure (11) PCR analysis of regenerated plants, agarose electrophoresis gels of PCR amplification products for PPT gene in SD 7 and SD 34 in respect.**