CHAPTER FOUR
RESULTS AND DISCUSSION

4.1. Callus induction of Maize

The immature embryos of Single cross 168 and SC10 were used those length was 1.6-2.0 mm. The embryos were selected and cultivated in D culture medium then transferred to N6E culture medium, after three times of cultivation (Figure 4). The present results produced three types of callus, embryo genic, non-embryo genic and organogenic callus. The present results are in accordance with those reported by (**Vassil *et al*., 1991; Armstrong and Green, 1985**) who found the same results.

Every 21 days Type II was selected and transferred to fresh medium (Figure 5 & 6) that when cultured *in vitro* can originate both Types embryogenic calluses. The results indicated clearly that, Type I callus have different morphological characteristics such as formed by hard, compact and yellowish tissue, these morphological characteristics effect of the viability to regenerate news plants, on the other hand the results showed that, Type II callus have different morphological characteristics such as soft, friable, highly embryogenic and able to regenerate a higher number of plants than Type I callus. Our results proved that only Type II embryogenic callus can regenerate, on the other hand **Brettschneider *et al*., (1997) and Frame *et al*., (2006**) found that both types of calluses can be used to generate for tissue culture.

Results showed that hybrid 168 is more efficient than SC10 in callus regeneration and response for sub-culture. The higher alive calluses were (88.17 and 41.0) in 168 and SC10 hybrids, in respect. The number of dead calluses in variety SC10 was (26.67) compared with variety 168 was (7.17) as shown in Figure 5 and 6. The main conclusions from these results that variety 168 could be used for transformation more efficiency SC10 based on their callus morphological characteristics.

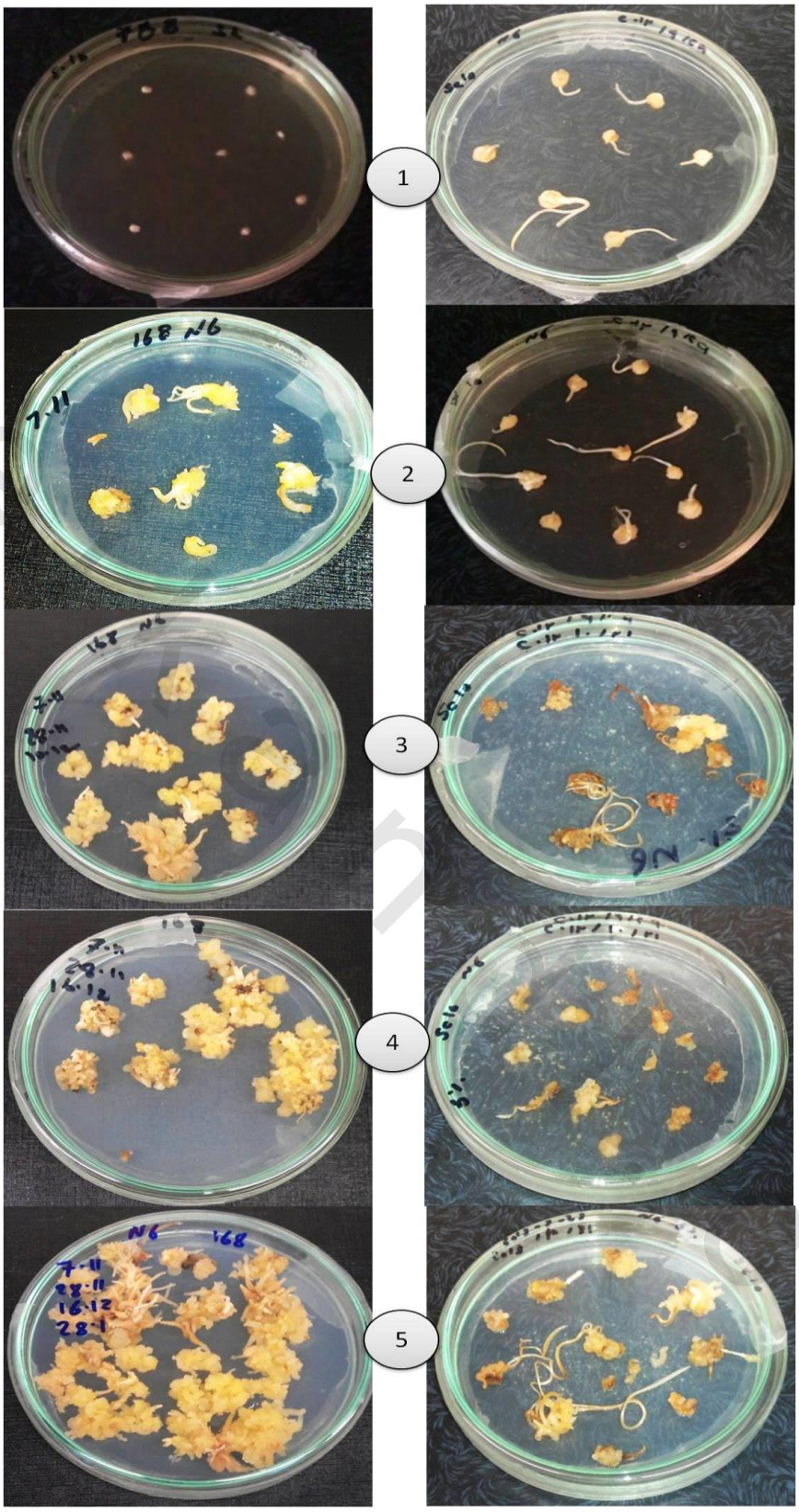

Figure (4) The different growth stages for embryo cultured on medium D and N6E on variety SC10 (Right) and SC168 (Left) as follow: (1) planting of immature embryo; (2) embryo growth and forming; (3);Alive and dead callus (4) callus induction and (5) sub-culture of callus.

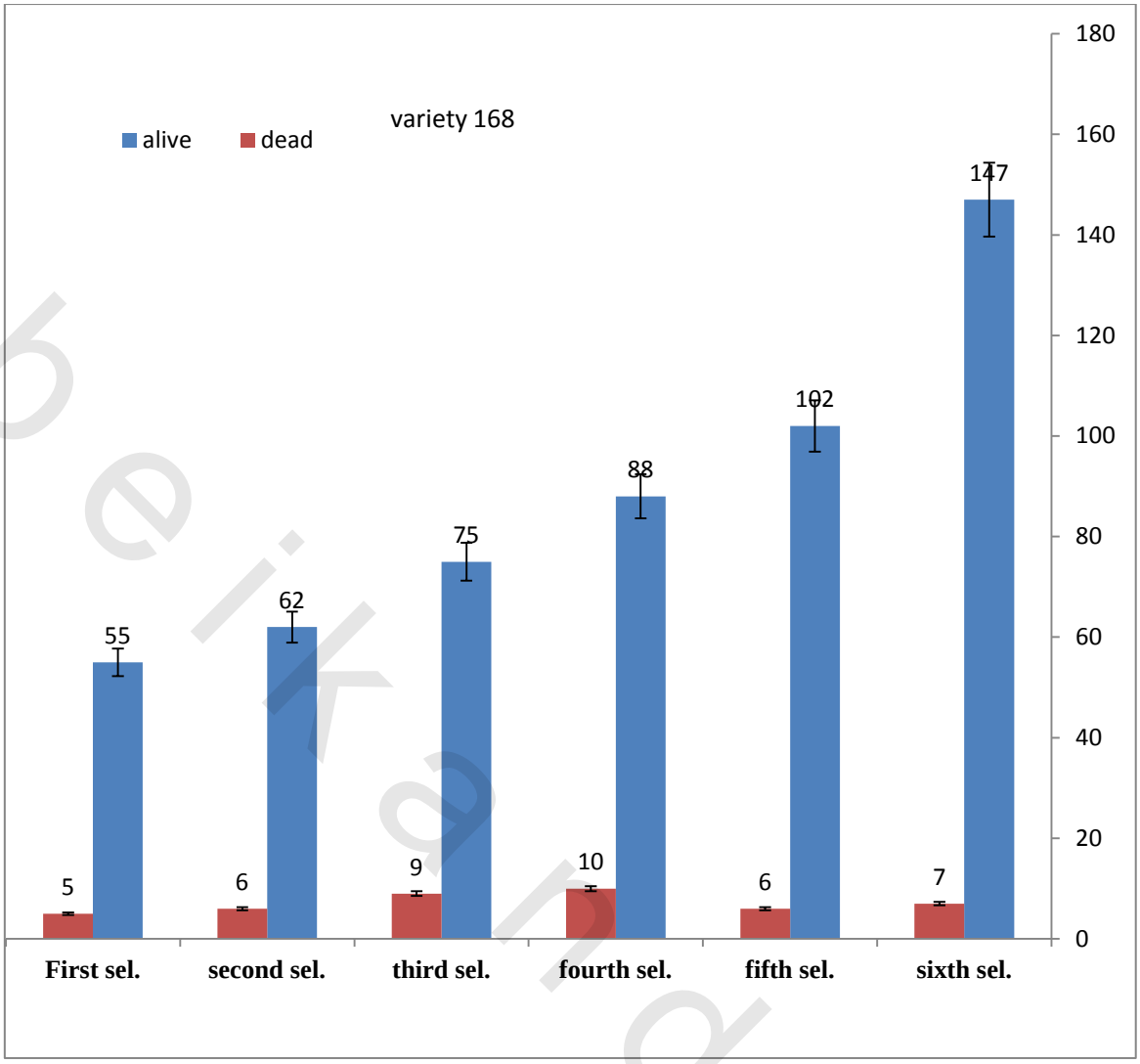

Figure (5). Different selection for alive and dead calluses number on variety SC168 using N6E medium

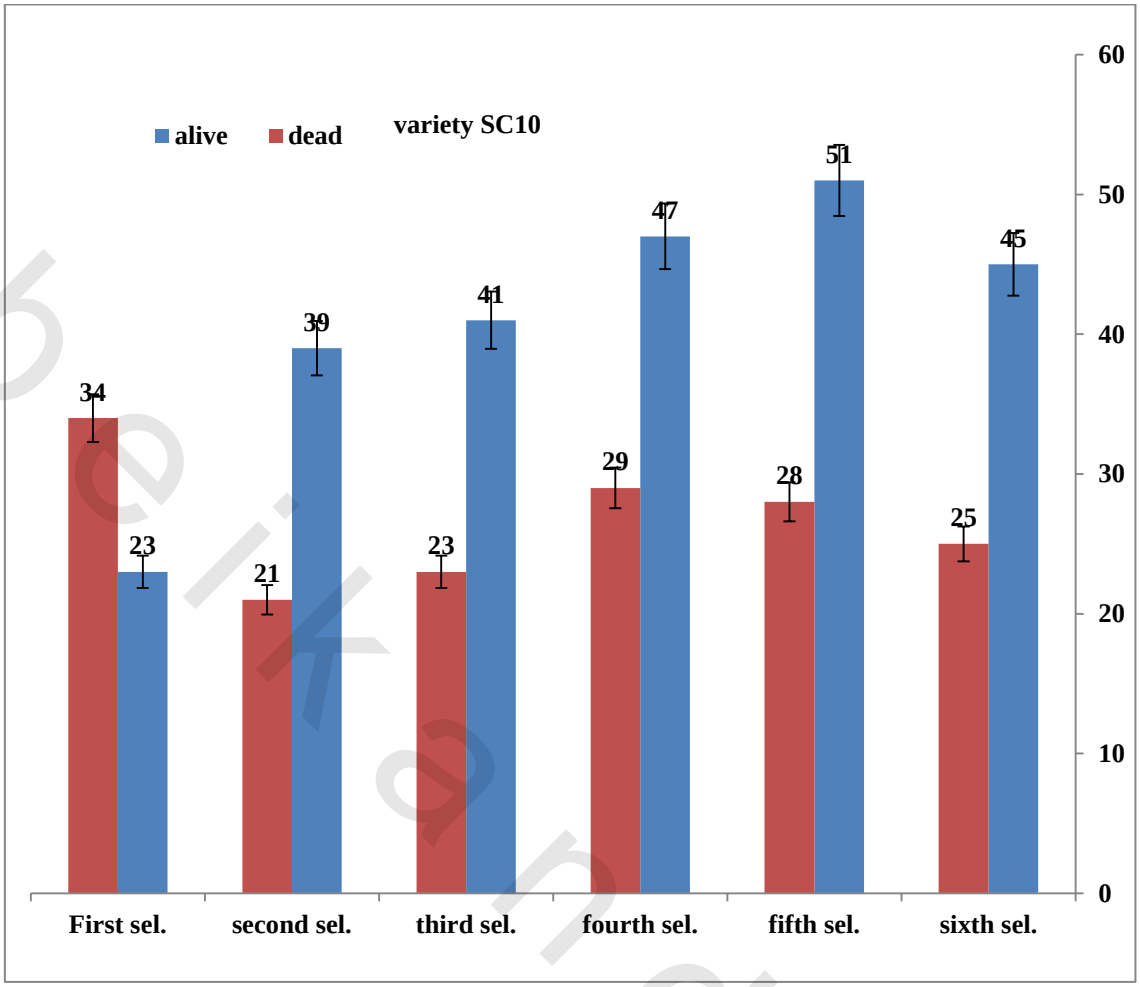

Figure (6) Different selection for alive and dead calluses number on variety SC10 using N6E medium.

Data in Figure 7 and 8 showed the overall of alive and dead callus for the selected hybrids SC10 and 168 in respect. The highest values for alive callus were 147 and 51 in SC168 and 10, respectively. While the lowest values were 55 and 23. The overall of alive callus was 88.17 and 41.0 and dead callus was 26.67 and 7.17, in respect. SC168 was more efficient than SC10 and the percentage of dead callus was 8% compared with the other variety was 65% (Table 2)

The results showed that, callus was initiated on N6 medium supplemented also; results indicated that, induction of primary callus ranged between 0 and 93%. Generally, three types of callus were formed: embryo genic, non-embryo genic and organogenic callus. The embryo genic callus was formed within two weeks of culture in callus maintenance medium. Induction of embryo genic callus ranged between 0 and 5%. Somatic embryos were matured on N6 medium supplemented with 6% sucrose and 1 mg.dm-3 NAA.

Somatic embryogenesis is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a somatic embryo. These characteristics have designated somatic embryogenesis into a model system for the study of morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants.

Every 40 days for three times we calculated the dead and alive callus as shown in Table 2. Almost 50% after 40 days of the callus were dead in SC10 and on the other hand in SC168 were nearly 90% of the callus were alive. The maximum number of alive callus were 28 in SC10 forwared by 26 for SC168.

For the second read (80 days) results in Table 2 showed the alive callus increasing while the dead callus were deceasing. For example the highest number was 18.71 in SC168 compared with 11.65 in SC10 (Table 2). 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 15.01 in SC168 compared with 9.68 in SC10. (Table 2)

Table (2) Dead and a live callus of two in breed maize line used in the current resarsch

Inbreed lines		**Sc10**		**Ss168**	
Reading		**Dead**	**A live**	**Dead**	**A live**
Read 1 (40 days)	**Average**	9.38±0.98	9.46±1.0	1.41±0.32	17.06±2.1
	Maximum	22	28	10	26
	Minimum	0.0	0.0	0.0	1
Read 2 (80 days)	**Average**	4.31±0.67	11.65±1.65	1.69±0.43	18.71±2.33
	Maximum	15	27	7	25
	Minimum	0.0	2	0.0	9
Read 3 (120 days)	**Average**	2.94±0.21	7.94±0.77	3.8±0.12	9.26±**1.23**
	Maximum	12	16	11	21
	Minimum	0.0	0.0	0.0	0.0
Overall		5.54±1.33	9.68±1.54	2.3±0.02	15.01±2.0
Total		**15.22**		**17.31**	
Percentage		**36.39**	**63.60**	**13.28**	**86.71**

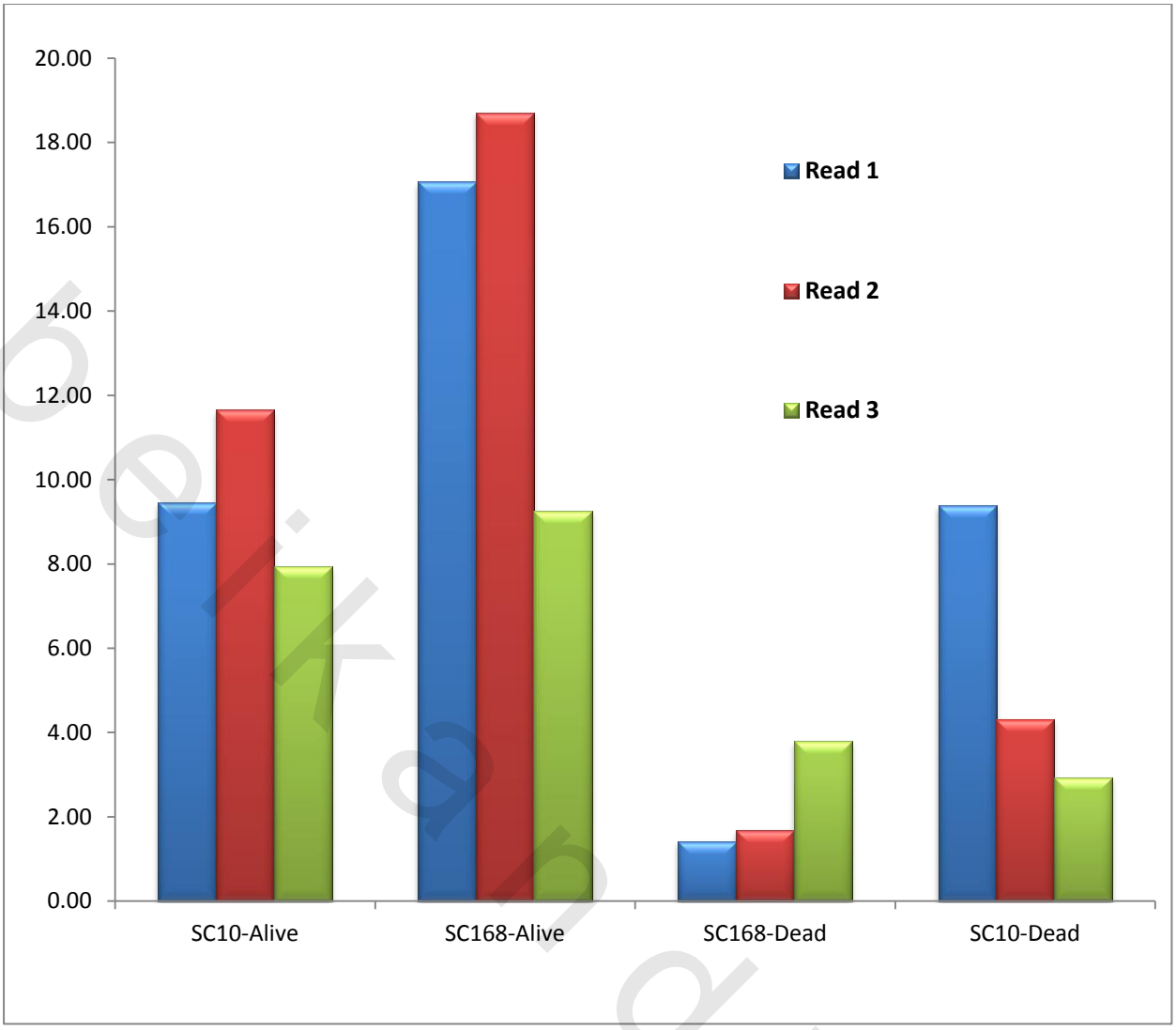

Figure (7) different values of dead and a live callus during the three reading

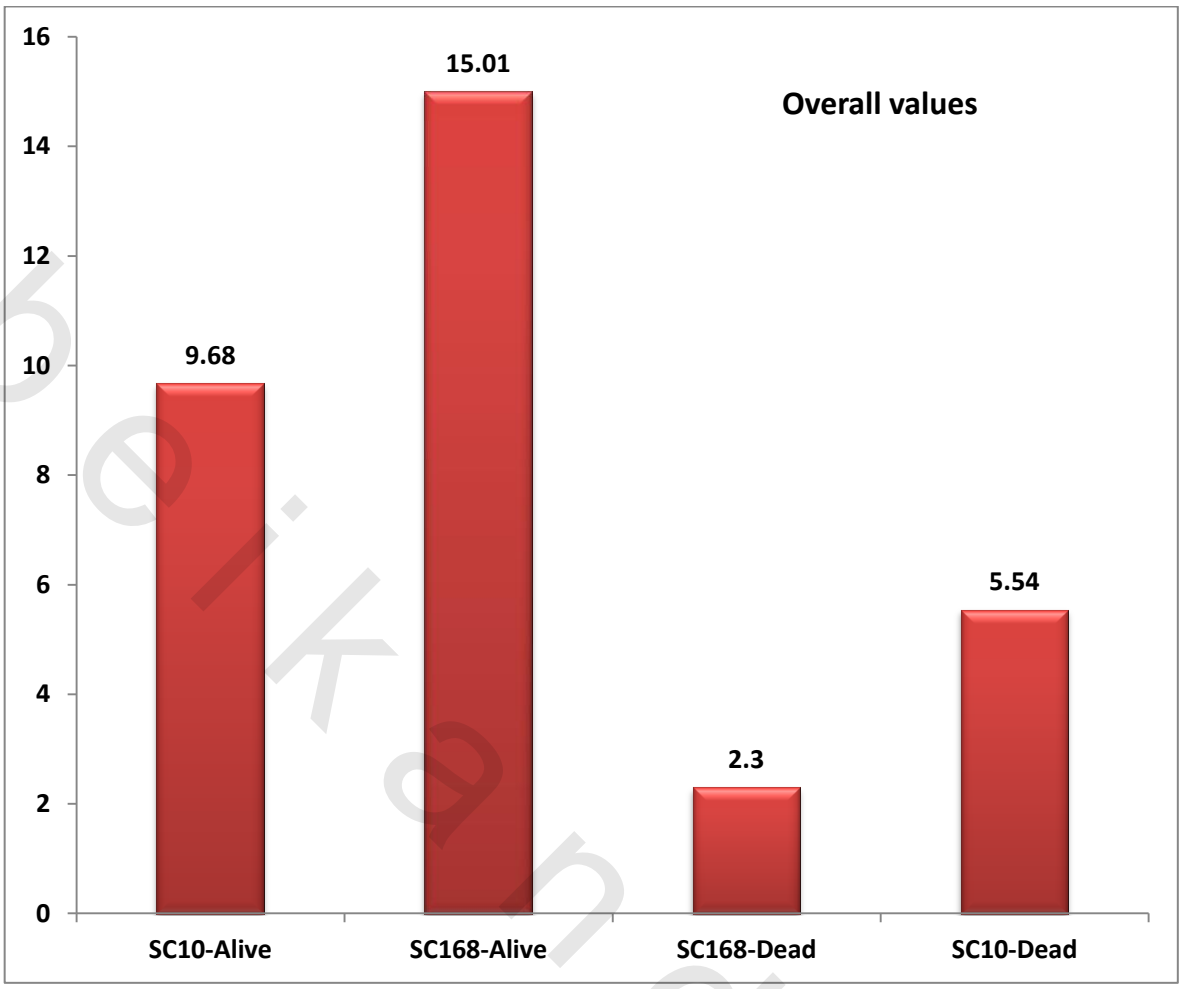

Figure (8) Overall values of dead and a live callus for SC10 and SC168 hybrids lines

4.2. Effect of callus morphology on transformation efficiency:

The immature embryos of SC10 and SC 168 in length was 1.6-2.0 mm. The embryos were selected and cultivated in D culture medium, then transferred to N6E culture medium. After 3 times of cultivation, two types of callus were produced.

The present results are in accordance with those reported by (**Vassil et al., 1991; Armstrong and Green, 1985**) who found that Type I compact, white that failed to regenerate, Type II friable, yellowish and able to regenerate. Type II was selected and transferred for particle bombardment.

When cultured *in vitro* can originate Type I or Type II embryogenic calluses. Type I callus is formed by hard, compact and yellowish tissue, usually unable to regenerate plants. Type II callus is soft, friable, highly embryogenic and able to regenerate a higher number of plants than Type I callus. Type II embryogenic callus can regenerate only. On the other hand (**Brettschneider *et al.*, 1997; Frame *et al*., 2006)**, found that both types of calluses can be used to generate transgenic plants.

4.3. Effect of osmotic treatment on transformation efficiency:

An important aspect in transformation via biolistics is the damage to the target tissue during microparticle penetration into the cell. To minimize this problem, the target cells are usually plasmolysed by an osmatic treatment and increase stable clone recovery (**Vain *et al*., 1993, Brettschneider *et al*., 1997**) so callus were put on osmoticum medium for 4 hours before bombardment. For bombardment, 900 and 1100 psi were used to shot calluses (single and double shout).

The results indicated that double shot were more efficient than single shot. However, the differences between treatments were not significant according to Chi square test at p0.05 (0.3327). (Table 3,4 and 5) Clearing by demonstrates that the number of living calluses decreased with double shot but these living calluses showed positive results for PCR and are more efficient than single shot.

El-itriby *et al*., (2003) concluded from the transient GUS expression that, in the co-transformation experiments, the use of osmotic pre- and post-treatment with acceleration pressure of 1100 psi and double shots per plate is most efficient treatment.

4.2.3. Selection of transgenic maize plants over expressing ASA2

PCR analysis was conducted on all of the plants regenerated from immature embryo transformation. It was observed 810bp band of ASA2gene (Fig. 10) and 790 bp of ASB gene (Fig.10).

Cho *et al*., (2004) showed that, the tobacco- feedback –insensitive ASA2 gene can be inserted into legume hairy roots (*A. sinicus* and soybean) and be expressed to produce feedback-insensitive AS activity that leads to increased free Trp levels and resistance to the Trp analog 5MT. The Trp analog 5MT and 6MT inhibit AS enzyme activity and plant growth. Over expression of the ASA2 gene results in resistance to 5MT in *E.coli* **(Song *et al*., 1998)** and transgenic *A. sinicus* **(Cho *et al*., 2000**).

The expression of ASA2 in plant tissues leads to increased levels of free Trp, which could be desirable since Trp. is an essential amino acid required in the diets of humans and non ruminant animals. To determine the over expression of the feedback- insensitive ASA2 had on Trp. production, free Trp levels in the T0 transgenic plant leaves were measured.

The high- expresser lines 9 and 12 contained > 12-16 times, line 1 show the Trp. level as wild type (Fig 6). In this report, we demonstrate that a feedback-insensitive ASA2 gene can be used as a selectable marker for the production of transgenic maize plants. Since maize expressing ASA2 resistant to 6MT, thus ASA2 may be an effective selectable marker gene for use with many different species.

Table (3) number of calluses living and dead on selection medium N6S + 6MT 75µM

# of calli	a live	dead	% of live calli
6MT 75 with double 900			
29	21	8	72.4
27	21	6	77.8
25	23	2	92.0
81	**65**	**16**	**80.2**
6MT with 75 doublen 1100			
27	23	4	85.2
26	22	4	84.6
24	20	4	83.3
77	**65**	**12**	**84.4**
Control			
33	11	22	33.3

Table (4) number of calluses living and dead on selection medium N6S + 6MT 100µM

# of calli	a live	dead	% of live calli
6MT 100 with double 900			
23	2	21	8.7
26	3	23	11.5
26	5	21	19.2
24	6	18	25.0
27	5	22	18.5
23	1	22	4.3
28	3	25	10.7
24	2	22	8.3
21	5	16	23.8
23	6	17	26.1
27	7	20	25.9
29	10	19	34.5
27	14	13	51.9
23	2	21	8.7
328	**69**	**259**	**21.0**
6MT with 100 doublen 1100			
33	8	25	24.2
31	7	24	22.6
24	1	23	4.2
31	3	28	9.7
29	6	23	20.7
33	12	21	36.4
29	9	20	31.0
210	**46**	**164**	**0.22**
Control			
33	11	22	33.3

Table (5) number of calluses living and dead on selection medium N6S + 6MT 125µM

# of calli	a live	dead	% of live calli
6MT 125 with double 900			
27	7	20	25.9
29	10	19	34.5
27	14	13	51.9
23	2	21	8.7
27	7	20	25.9
29	10	19	34.5
27	14	13	51.9
23	2	21	8.7
27	7	20	25.9
29	10	19	34.5
6MT with 125 doublen 1100			
22	9	13	40.9
27	10	17	37.0
27	9	18	33.3
29	12	17	41.4
105	**40**	**65**	**38.1**
Control			
39	13	26	33.3

2A

Figure (9) Morphology of different types of callus from left to right 75, 100 and 125 µM 6MT after bombardment

Figure (10) PCR analysis of regenerated plants. Agarose electrophoresis gels of PCR amplification products for ASA2 gene in SC 10 and SC 168 in respect.