CHAPTER IV

Results

4.1 Regeneration and callus formation of *Asparagus officinalis*

Generally callus formation and plant regeneration was easily obtained. Callus normally formed from the second week of inoculation. Regeneration was achieved from the nodal explants cultured on MS medium supplemented with BAP and NAA.

4.1.1 Effect of plant growth regulators on callus formation

So far, no study has been reported on *in vitro* propagation, callus culture and secondary metabolites for this plant. This study describes the basic procedures for the establishment of *in vitro* propagation of *Asparagus officinalis*.

Growth regulator composition on explants of *Asparagus officinalis* cultured on MS basal medium supplemented with auxins, cytokinins and auxin-cytokinin combination showed distinct morphogenic responses. Callus was induced in many of the 97 media in light and dark conditions as from the second week of inoculation. The calli proliferated very fast to form compact masses that ranged from pale yellow to green in color for light condition (Figure 4.1) and white color for dark condition (Figure 4.2).

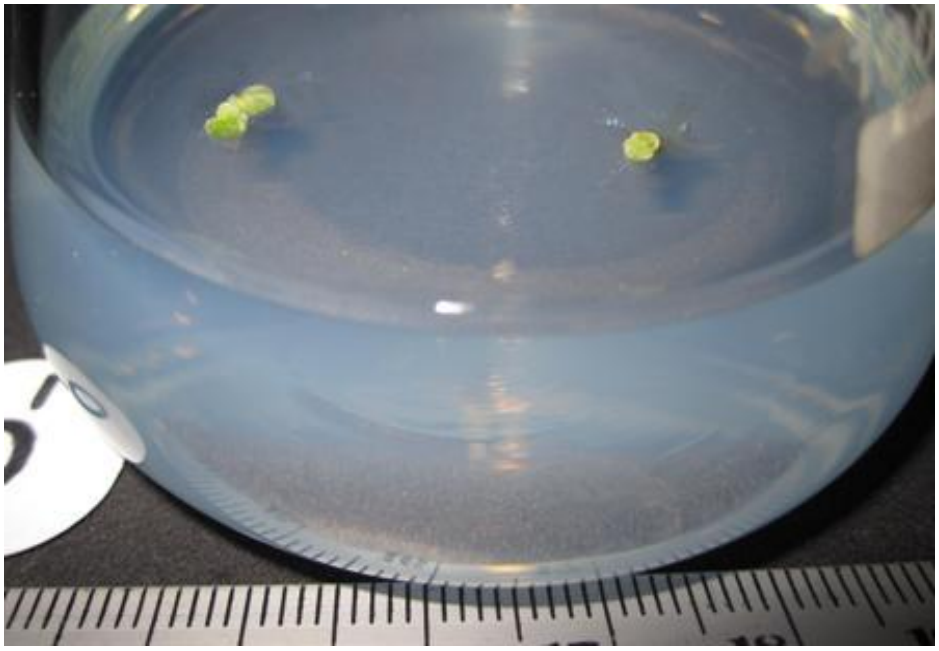

Figure 4.1 (I): Callus formation under light condition, in MS medium supplemented with 0.8 mg/L BAP + 0.1 mg/L NAA, after 3 weeks of culture

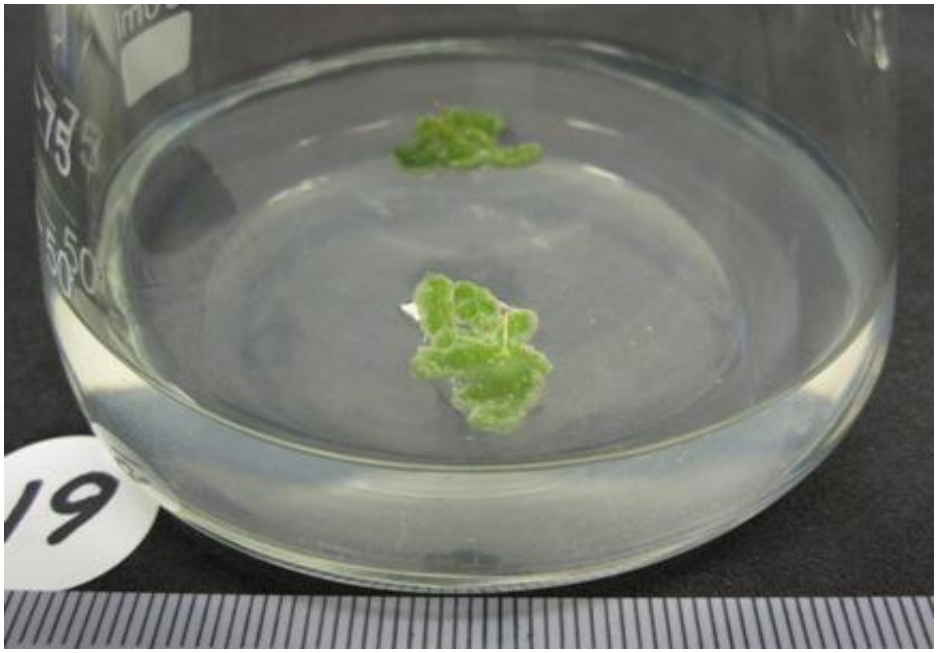

Figure 4.1 (II): Callus formation under light condition, in MS medium supplemented with 0.8 mg/L BAP + 0.1 mg/L NAA, after 6 weeks of culture

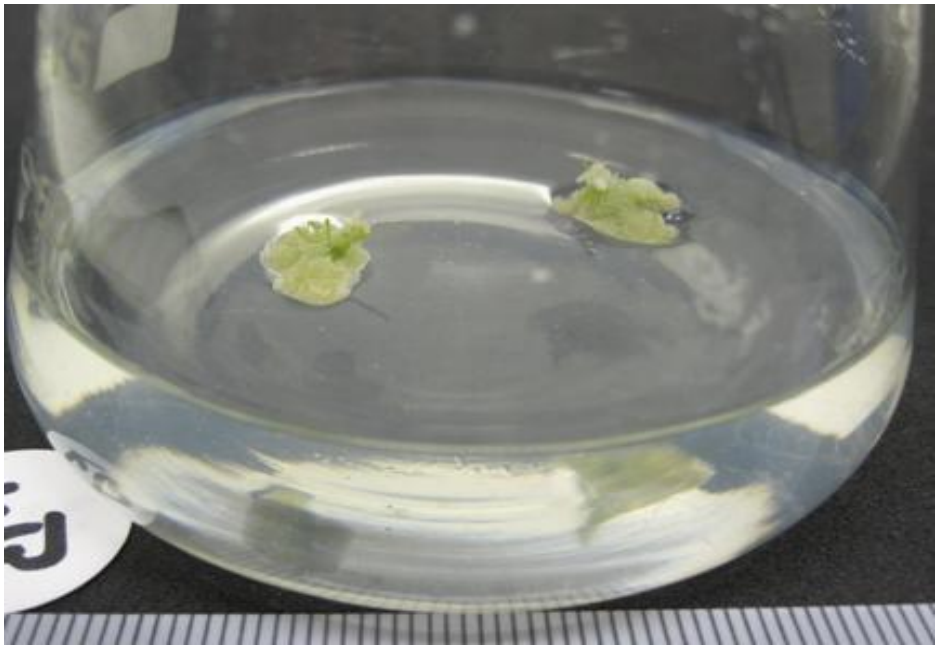

Figure 4.2 (I): Callus formation under dark condition, in MS medium supplemented with 0.4 mg/L Kn + 0.2 mg/L IBA, after 3 weeks of culture

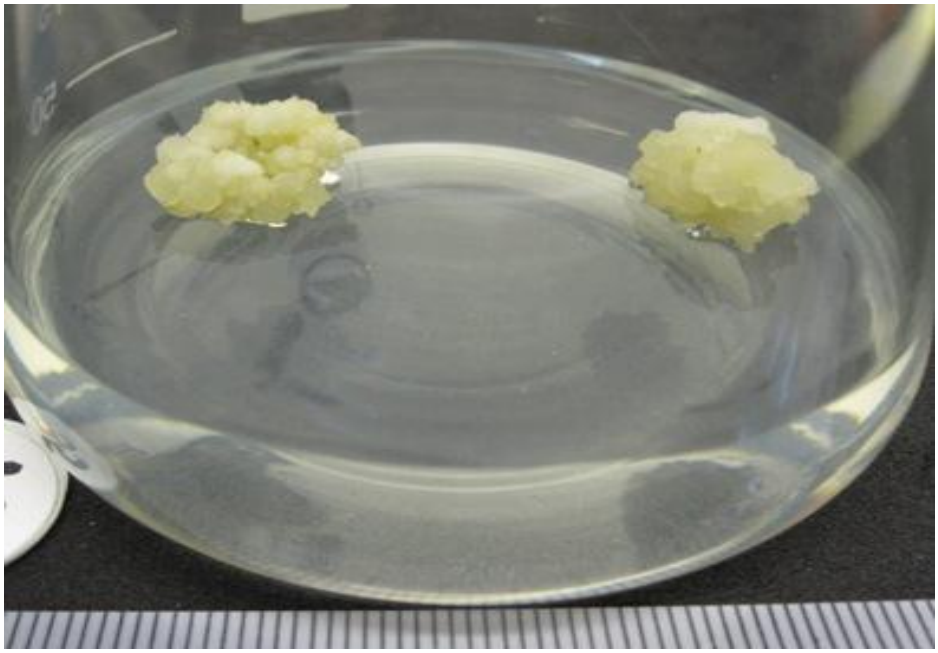

Figure 4.2 (II): Callus formation under dark condition, in MS medium supplemented with 0.4 mg/L Kn + 0.2 mg/L IBA, after 6 weeks of culture

After 6 weeks, callus was initiated in many cultures but the best results with 100% of callus formation were observed in the following treatments:

Under dark condition (Figure 4.3 and 4.5):

- 1.5mg/l BAP + 0.1mg/l NAA
- 0.2mg/l BAP + 0.2mg/l NAA
- 0.2mg/l BAP + 0.3mg/l NAA
- 0.4mg/l Kn + 0.1mg/l IBA
- 0.6mg/l Kn + 0.1mg/l IBA
- 0.8mg/l Kn + 0.1mg/l IBA
- 1.0mg/l Kn + 0.1mg/l IBA
- 1.5mg/l Kn + 0.1mg/l IBA
- 0.4mg/l Kn + 0.2mg/l IBA
- 0.6mg/l Kn + 0.2mg/l IBA
- 1.5mg/l Kn + 0.2mg/l IBA
- 0.6mg/l Kn + 0.3mg/l IBA
- 0.8mg/l Kn + 0.4mg/l IBA
- 1.0mg/l Kn + 0.4mg/l IBA
- 1.5mg/l Kn + 0.4mg/l IBA
- 0.8mg/l Kn + 0.5mg/l IBA
- 1.0mg/l Kn + 0.5mg/l IBA

Under light condition (Figure 4.4):

- 0.6mg/l Kn + 0.2mg/l IBA
- 0.4mg/l Kn + 0.3mg/l IBA
- 0.6mg/l Kn + 0.4mg/l IBA

In media with BAP+NAA, under light condition the highest percentage of callus formation was found as 75% (Figure 4.6).

In comparison of all treatments of two Cytokinins and two Auxins with control group, it was determined that the media should be supplemented with both cytokinins and auxins hormones for callus formation of *Asparagus officinalis* and the amount of cytokinin should be more than auxin.

Among the two groups of hormones (BAP + NAA, Kn + IBA concentrations), Kn + IBA was reported to be more efficient than BAP + NAA in promoting callus formation. Also, between dark and light condition, dark condition was found to be more efficient than light condition in promoting callus formation.

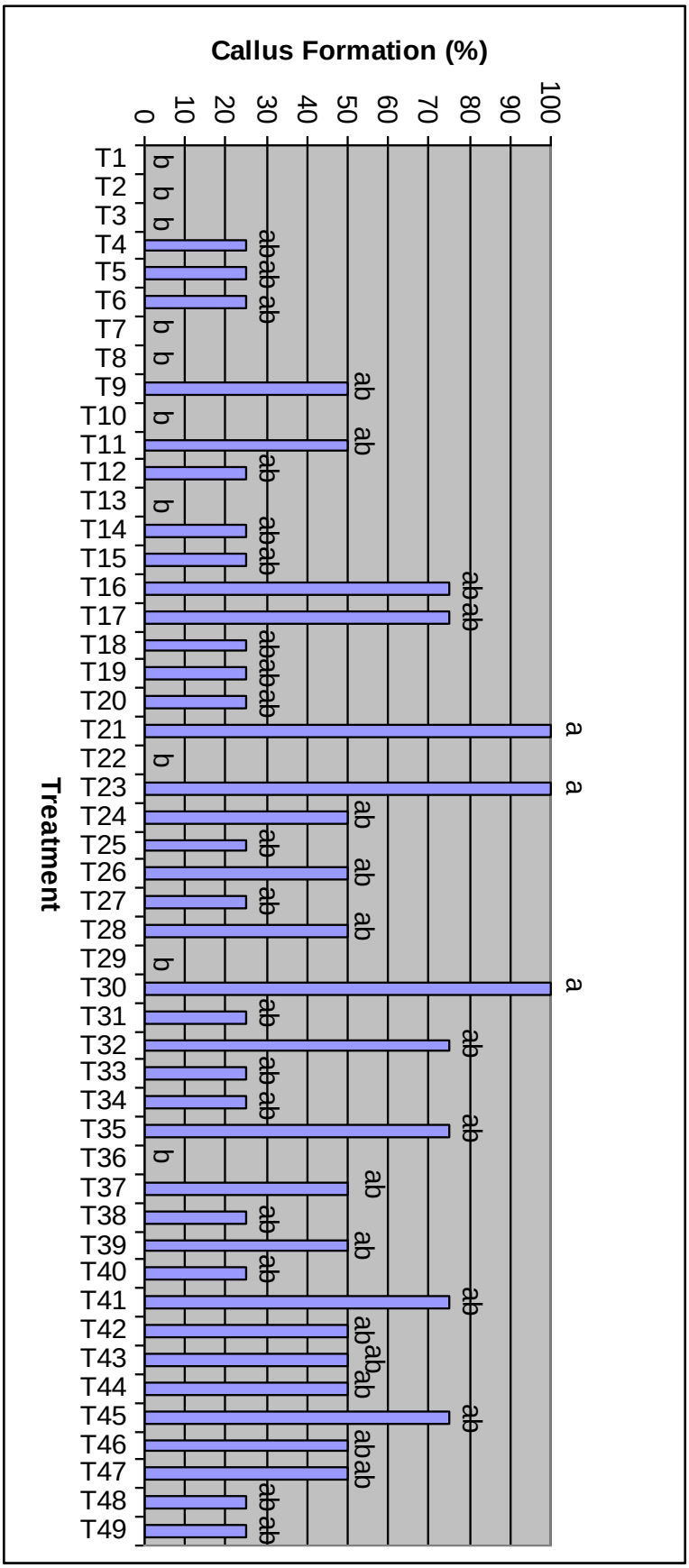

Figure 4.3: Percentage of callus formation in MS media with different concentration of BAP + NAA under dark condition, after 6 weeks

Figure 4.3 shows the percentage of callus formation in MS medium supplemented with BAP + NAA under dark condition. It can be seen from the results, 3 treatments showed the best result with 100% of callus formation. The lowest percentage of callus formation was obtained on the MS medium supplemented with BAP or NAA alone, not in combination. Increase in BAP + NAA concentration reduces the callus formation.

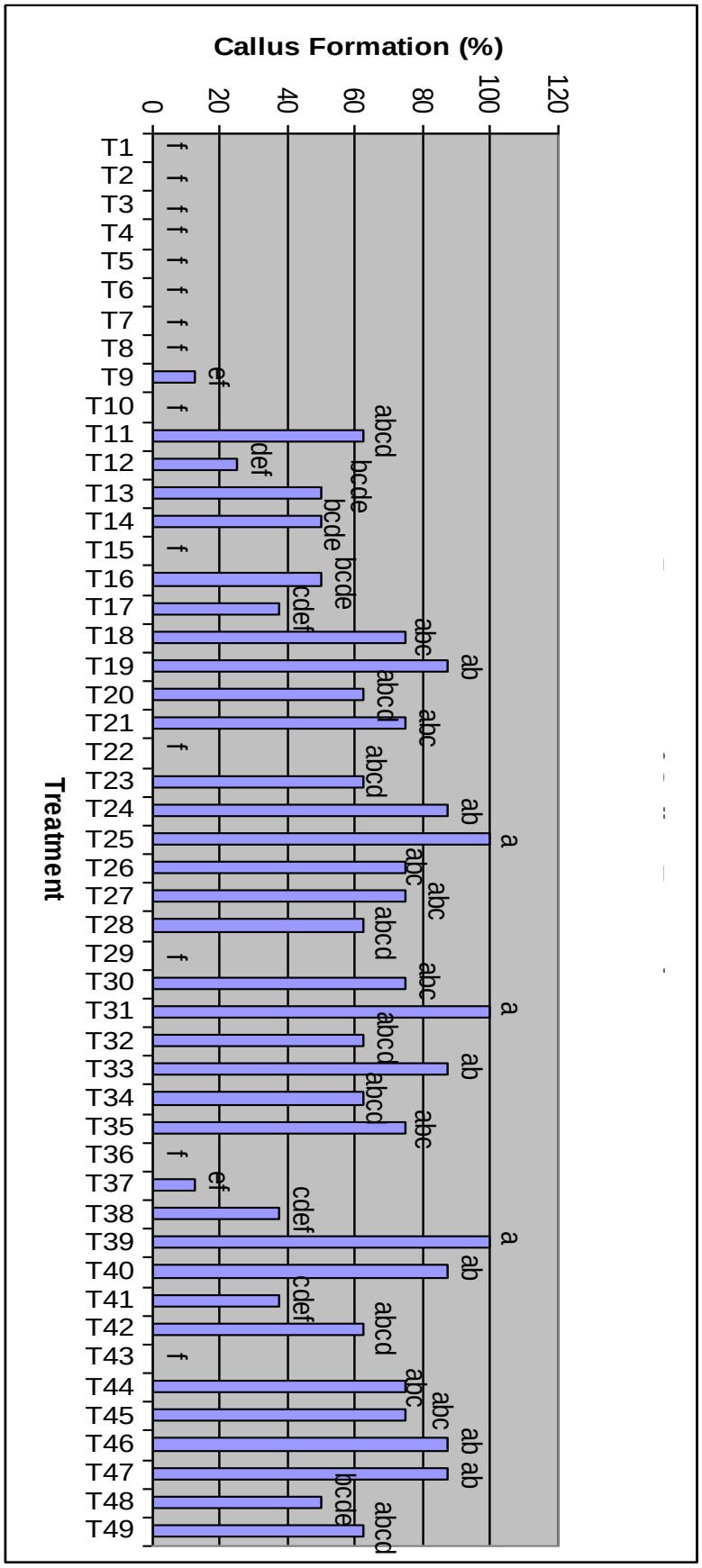

Figure 4.4: Percentage of callus formation in MS media with different concentrations of Kn + IBA under light condition, after 6 weeks

Figure 4.4 shows the percentage of callus formation in MS media supplemented with different concentrations of Kn + IBA under light condition. The highest frequency of callus formation (100%) in this segment was observed in 3 treatments (0.6 mg/l Kn + 0.2 mg/l IBA)(0.4 mg/l Kn + 0.3 mg/l IBA)(0.6 mg/l Kn + 0.4 mg/l IBA). The lowest percentage of callus formation (0%) was obtained on the MS medium supplemented with Kn or IBA alone, not in combination.

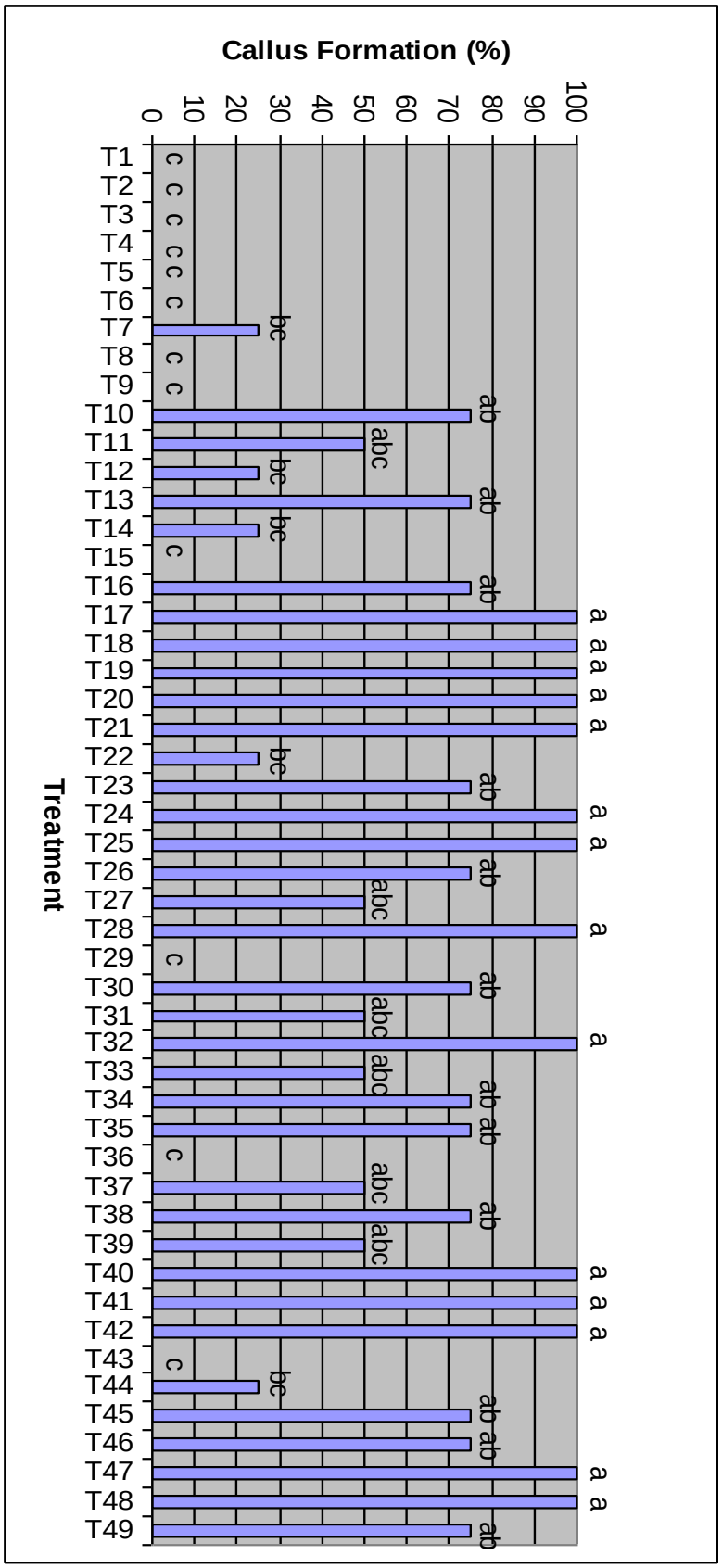

Figure 4.5: Percentage of callus formation in MS media with different concentrations of Kn + IBA under dark condition, after 6 weeks

Figure 4.5 shows the percentage of callus formation on MS medium supplemented with different concentrations of Kn and IBA under dark condition. Callus was formed in most treatments with different frequencies. Fourteen treatments gave 100% of callus formation. While there was no callus formation in the MS medium supplemented with Kn or IBA alone.

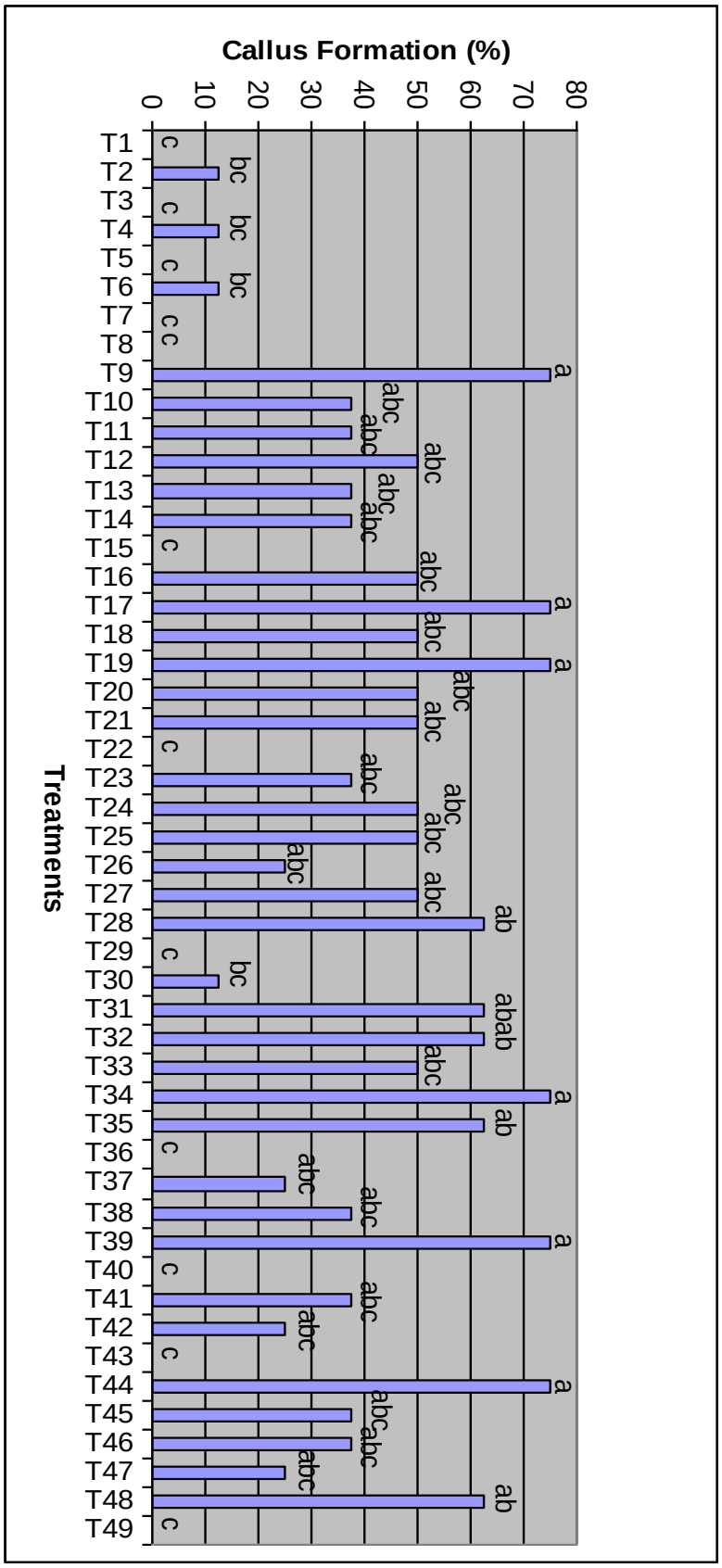

Figure 4.6: Percentage of callus formation in MS media with different concentrations of BAP + NAA under light condition, after 6 weeks

Figure 4.6 shows the percentage of callus formation in MS medium supplemented with BAP + NAA under light condition. Based on this figure none of the treatments show 100% of callus formation. The highest callus formation in this segment was found as 75% in 6 treatments (0.2 mg/l BAP + 0.05 mg/l NAA) (0.4 mg/l BAP + 0.1 mg/l NAA) (0.8 mg/l BAP + 0.1 mg/l NAA) (1.0 mg/l BAP + 0.3 mg/l NAA) (0.6 mg/l BAP + 0.4 mg/l NAA) (0.2 mg/l BAP + 0.5 mg/l NAA).

4.1.2 Effect of plant growth regulators on shoot regeneration of *Asparagus officinalis*

After 6 weeks of culture, mean number and length of shoots per explant were recorded. The best treatments with the highest average number of shoot (4.25) of size 4 mm or more per explant was obtained on the medium supplemented with 1.5 mg/l BAP and 0.05 mg/l NAA under dark condition (Figure 4.7) but the shoots were less developed, with abnormal thick, yellow color (Figure 4.8) compared with the shoots produced under light condition. In light condition the highest average number of shoot (3.63) of size 4 mm or more per explant was found on the MS medium supplemented with 0.8 mg/L BAP alone, not in combination with NAA (Figure 4.9).

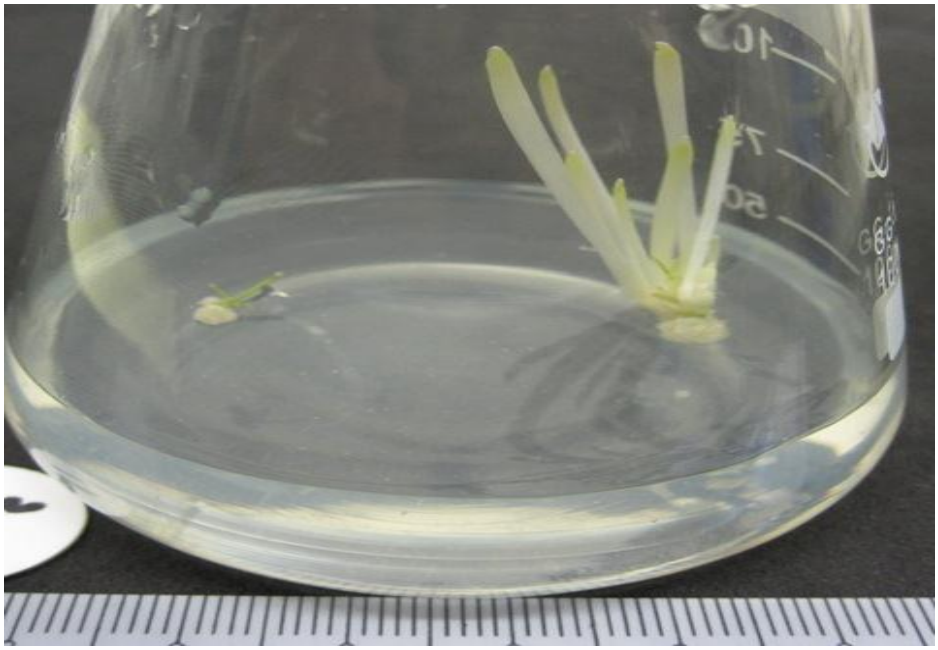

Figure 4.7: Shoot regeneration from cultured explant on MS medium supplemented with 1.5 mg/L BAP and 0.05 mg/L NAA under dark condition after 6 weeks

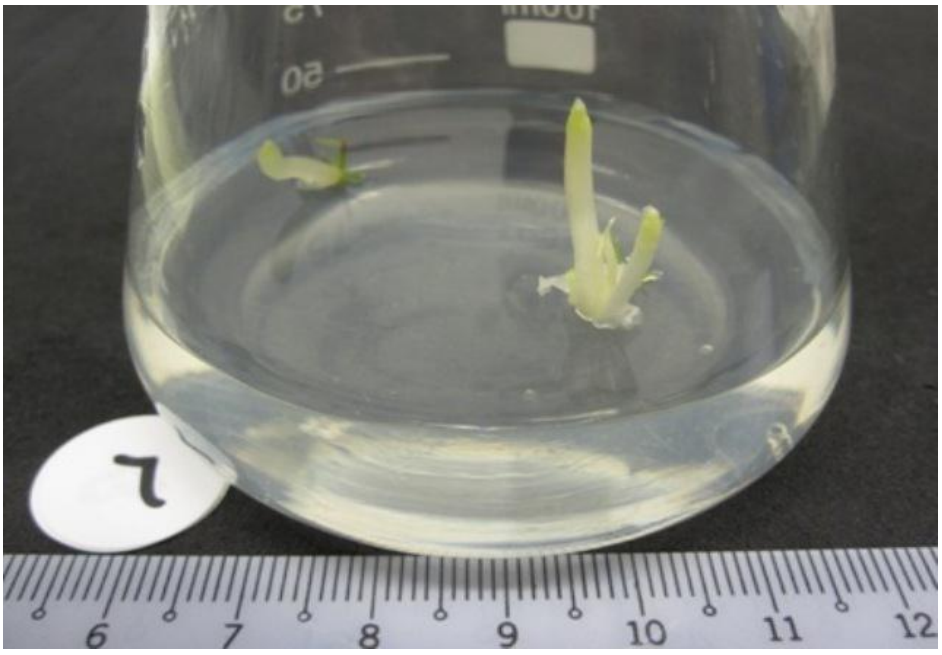

Figure 4.8: Shoot regeneration from cultured explant on MS medium supplemented with 1.5 mg/L BAP and 0.05 mg/L NAA under dark condition after 6 weeks

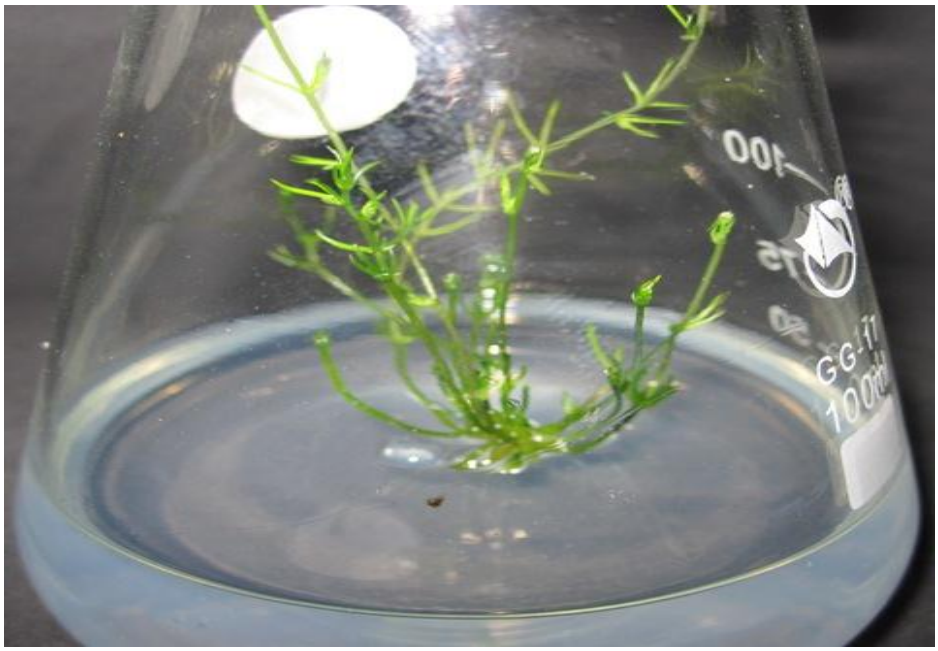

Figure 4.9: Shoot regeneration from cultured explant on MS medium supplemented with 0.8 mg/L BAP under light condition after 6 weeks

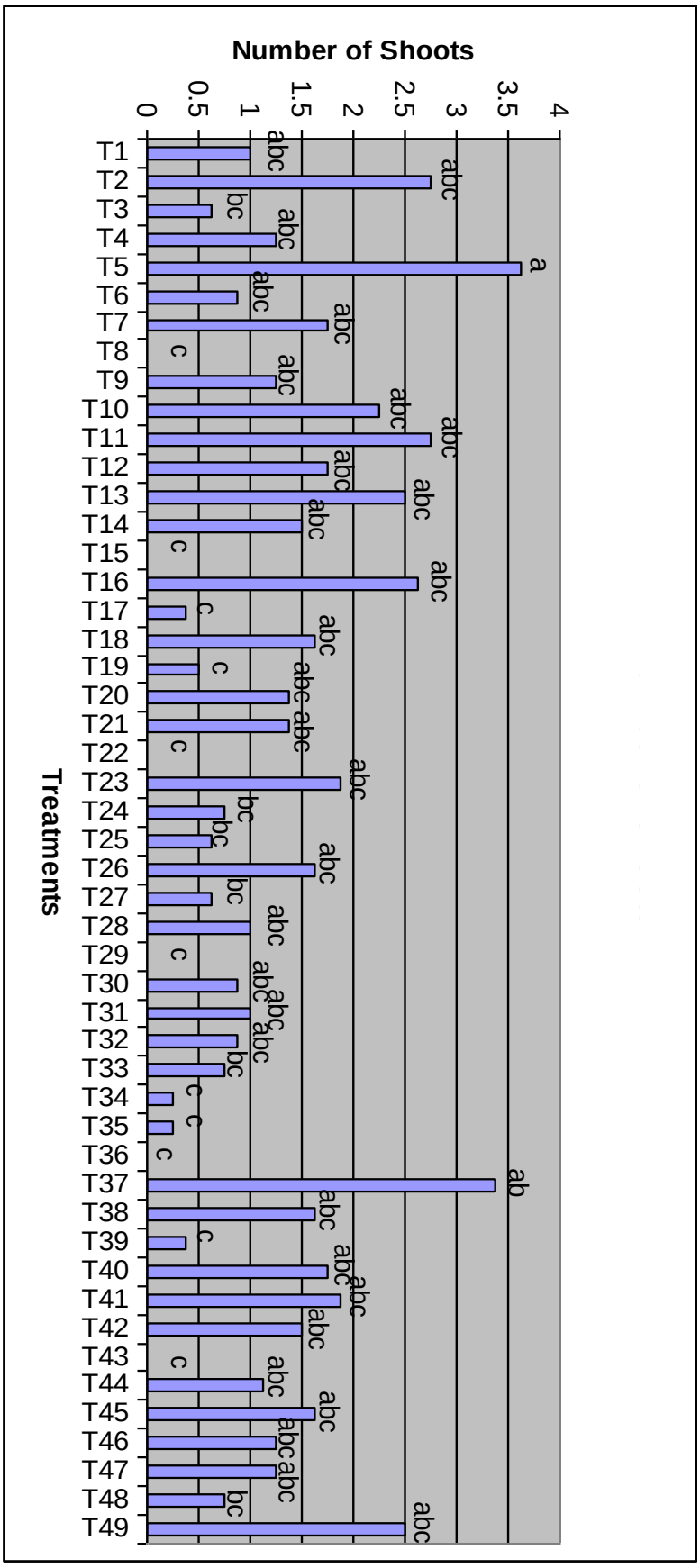

Figure 4.10: Mean number of shoots per explant in MS media with different concentrations of BAP + NAA uuder light condition, after 6 weeks

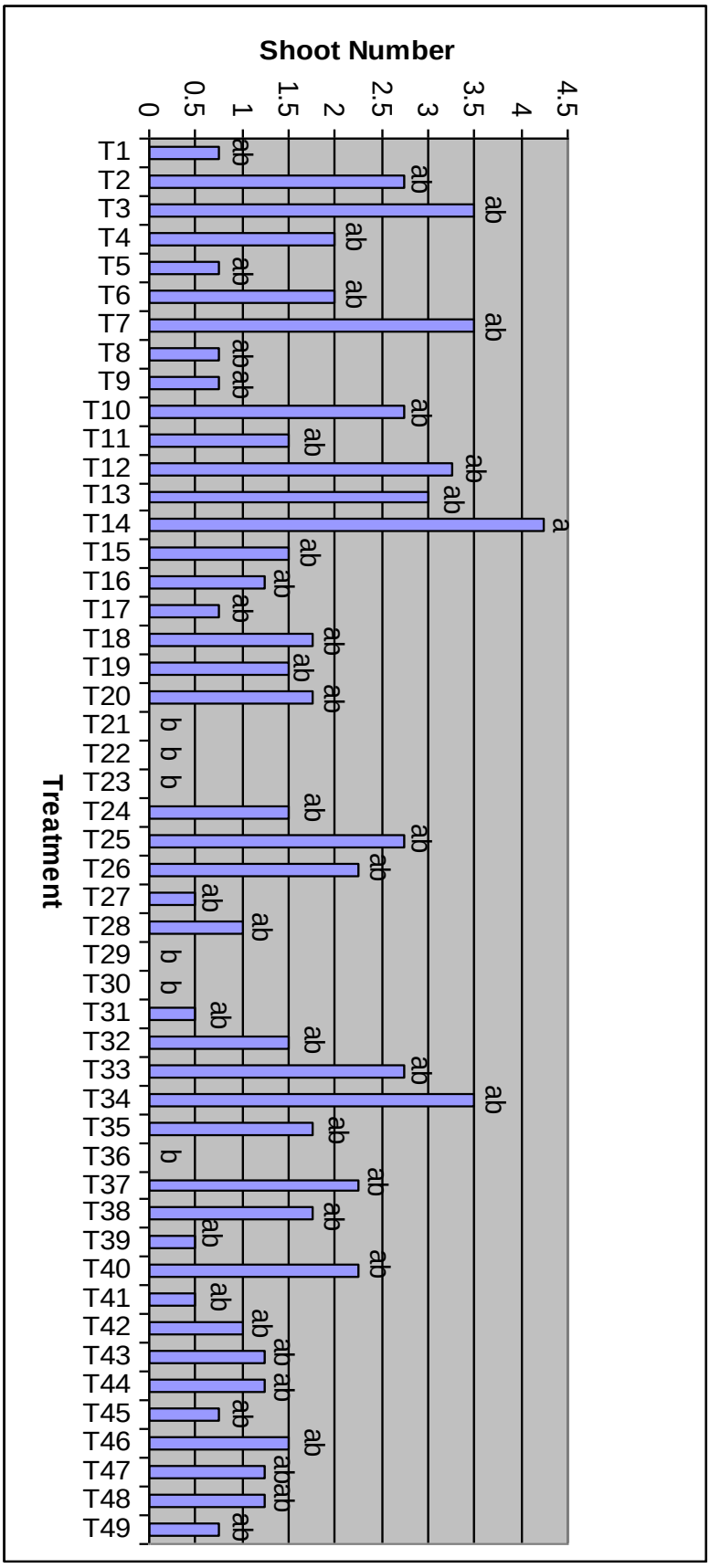

Figure 4.11: Mean number of shoots per explant in MS media with different concentrations of BAP + NAA under dark condition, after 6 weeks

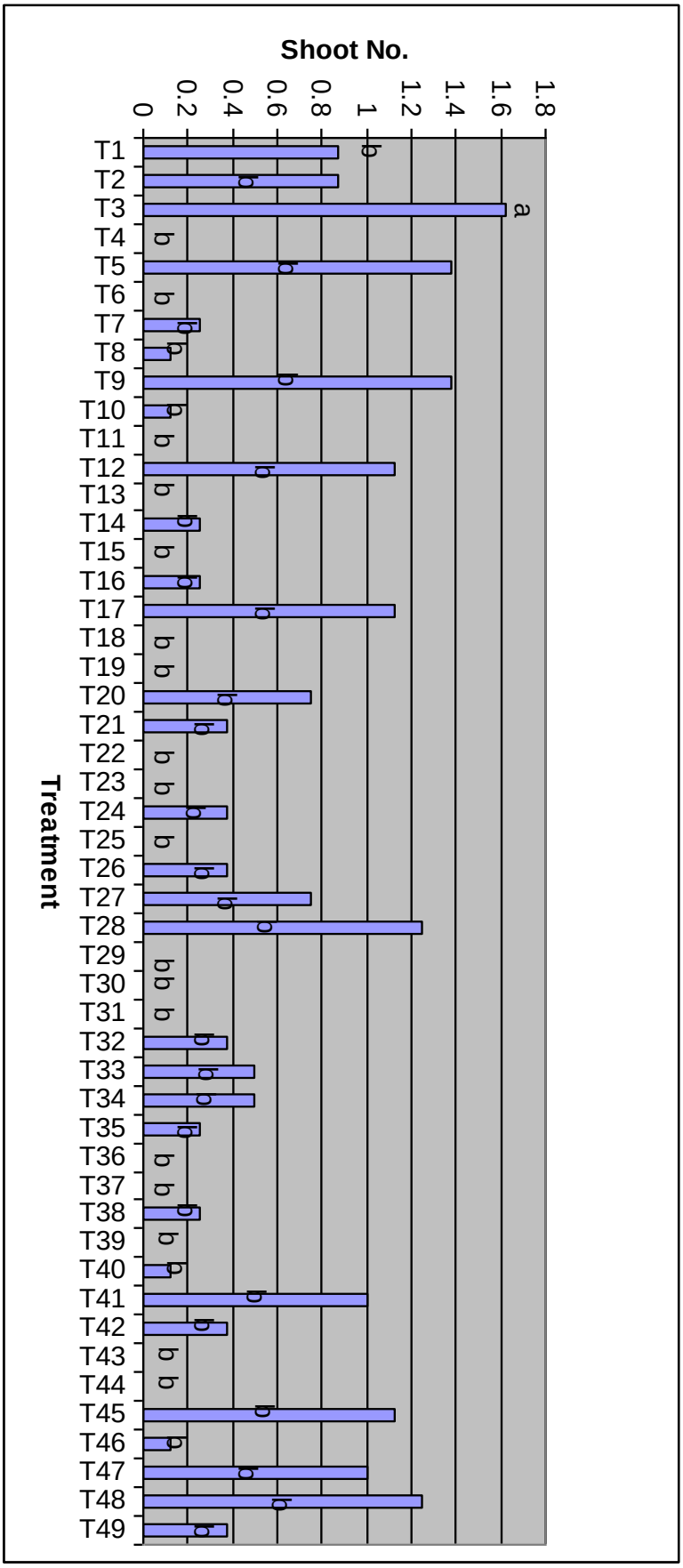

Figure 4.12: Mean number of shoots per explant in MS media with different concentrations of Kn + IBA under light condition, after 6 weeks

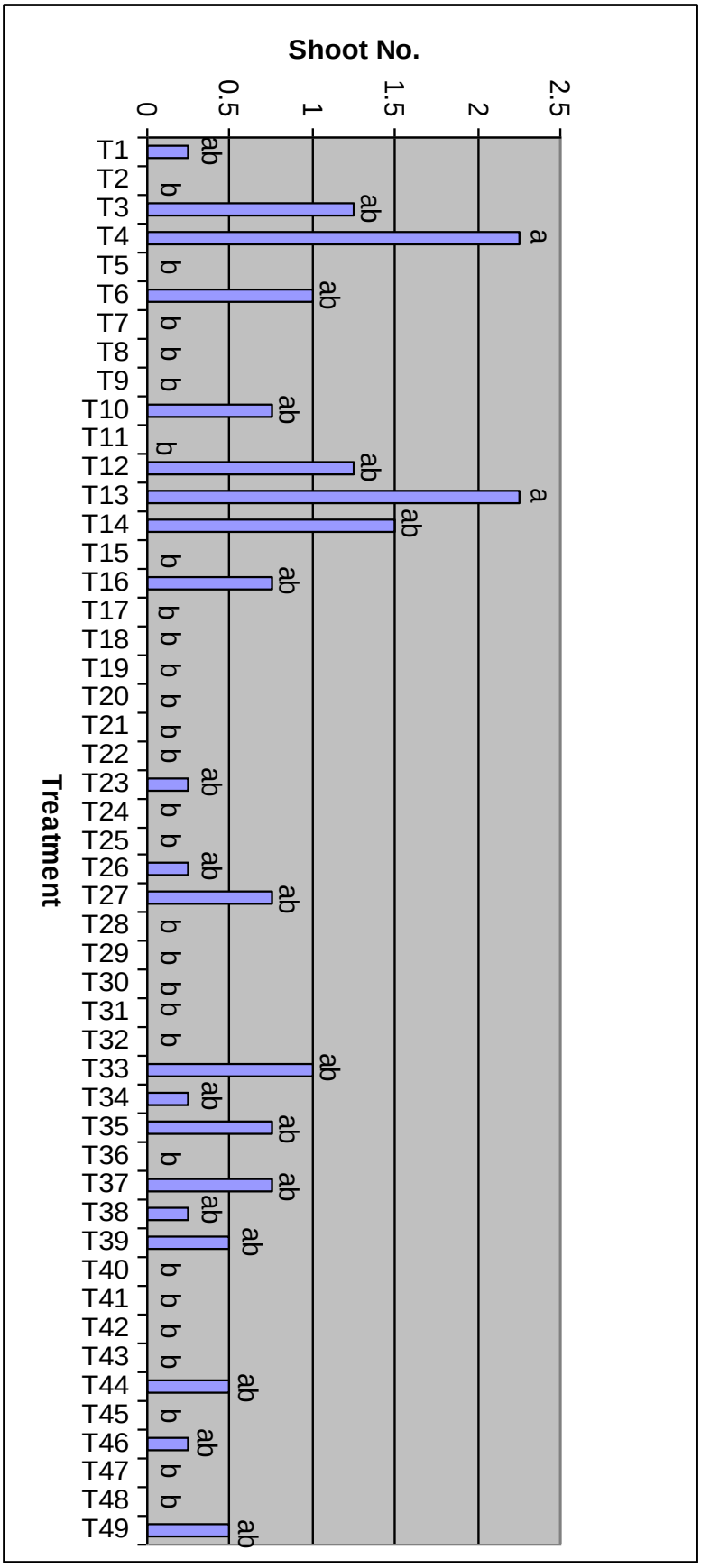

Figure 4.13: Mean number of shoots per explant in MS media with different concentrations of Kn + IBA under dark condition, after 6 weeks

4.1.3 Root development

In vitro induction of roots from growing shoots has been achieved in MS medium containing different concentrations of IBA. The regenerated shoots were very carefully rescued from the culture flasks, placed on a sterile petridish and were given the cut from the basal end of the shoots. Each of this shoot was then planted on rooting medium which consisted of different concentrations of IBA (0.1 mg/l, 0.2 mg/l, 0.3 mg/l, 0.4 mg/l, 0.5 mg/l, 0.75 mg/l, 1.0 mg/l, and 1.5 mg/l) for root initiation (Figure 4.14). Among the various concentration tested, 0.4 mg/l IBA showed the best result (2 roots per explant) where roots initiated after 6 weeks of culture. Figure 4.15 shows complete plantlets with elongated shoot and root systems.

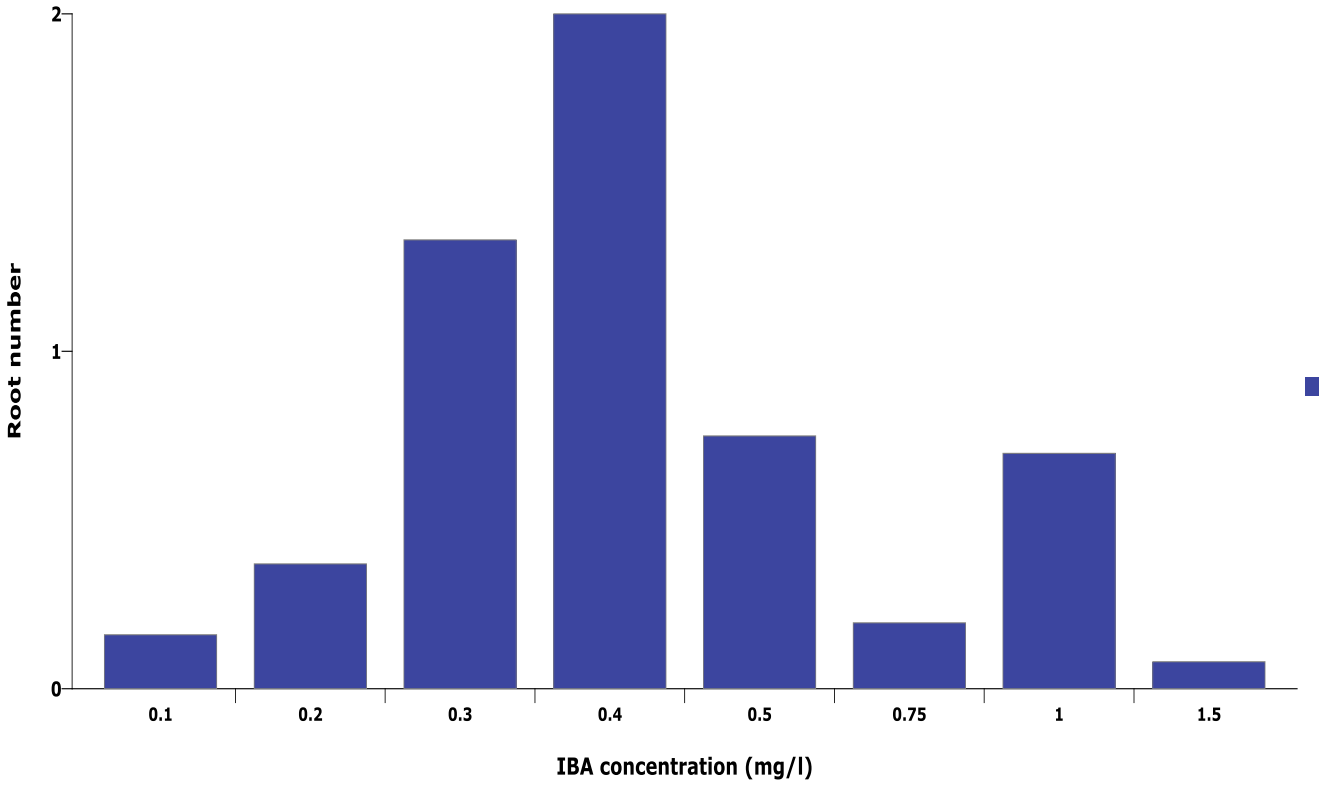

Figure 4.14: Mean number of roots per explant in MS media containing different concentrations of IBA, after 6 weeks

Figure 4.15: Root development in MS medium supplemented with 0.4 mg/L IBA after 6 weeks of culture

According to Yang and Clore (1974), rooting percentage increases when shoots are allowed more time on rooting media. In their studies they showed that shoots regenerated from stem segments cultured for 20 weeks had a higher rooting percentage (92.2%) when allowed to root for 8 weeks. The method used by Yang and Clore (1974), however, involved a longer culture period, which is a major drawback. Data obtained from Yang and Clore's (1974) study clearly indicates that the age of the shoots and the rooting time should be optimised to increase rooting frequency.

4.2 Antioxidant activity

4.2.1 SOD assay

Based on the result of SOD assay (Figure 4.16), *in vivo* plant extract obtained the highest inhibition rate (80.44%) among all the three examined plant extracts which is significantly different with both *in vitro* plant and callus extracts. Although, *in vitro* plant extract inhibition rate (35.42%) was slightly higher than callus extract (32.24%), there was no significant difference between them. All extracts used showed significantly lower inhibition rates compared to 1 mg/L ascorbic acid (98.35%) positive control.

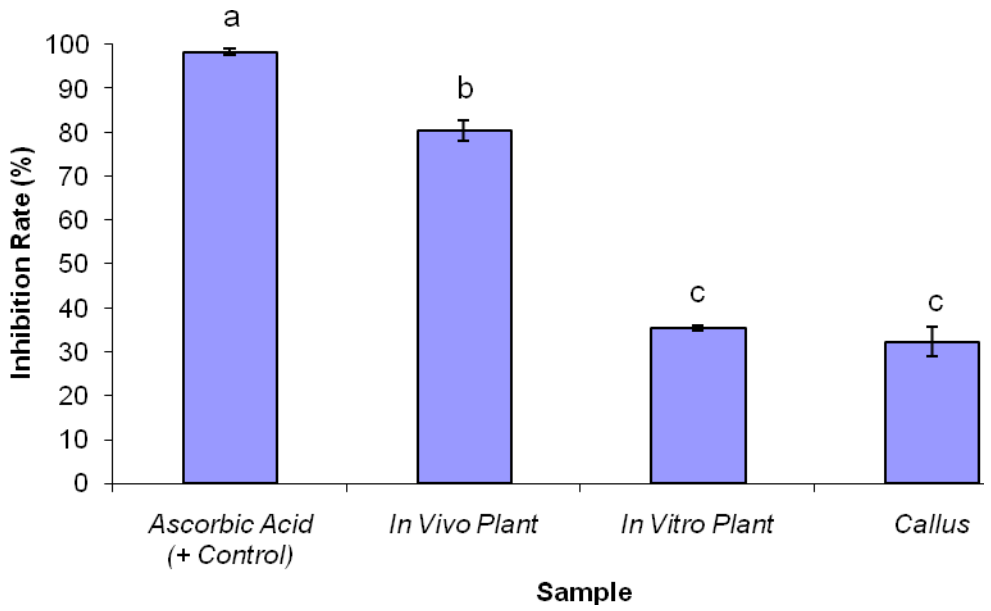

Figure 4.16: Antioxidant activity of plant extracts (*in vivo*, *in vitro* and callus) of *Asparagus officinalis* measured using superoxide dismutase (SOD) assay presented as percentage of inhibition rate. Ascorbic acid (1 mg/L) was used as the positive control. The data were analyzed by one-way ANOVA and the inhibition rate means of samples were compared using Duncan's Multiple Comparison Test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

4.2.2 Erythrocytes haemolysis assay

The result of the rabbit erythrocytes haemolysis assay is shown in Figure 4.17. The negative control (without pre-treatment) obtained a mean of 62.8% haemolysis, while ascorbic acid (1mg/L) could reduce the haemolysis percentage to 6.46% in positive control. All plant extracts examined in this study could reduce the erythrocytes haemolysis significantly compared to the negative control. *In vivo* plant extracts (14.47%) showed the highest erythrocytes haemolysis prevention compared to the *in vitro* (53.19%) and callus (54.47%) extracts. There was no significant difference between the rates of hemolysis reduction by *in vitro* plant and callus extracts while both of them were significantly different with *in vivo* plant extract.

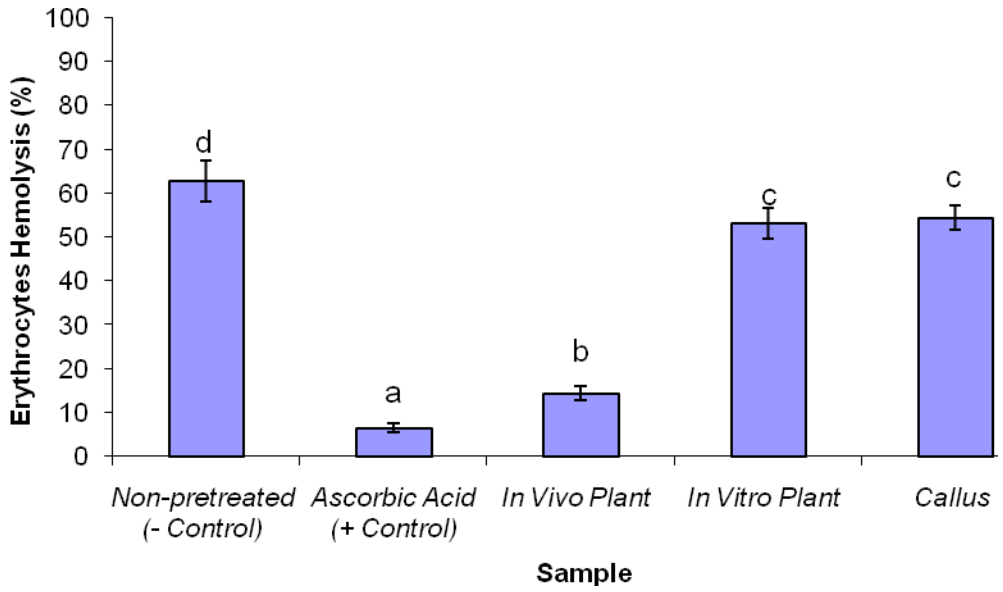

Figure 4.17: Antioxidant activity of examined plant extracts (*in vivo* plant, *in vitro* plant and callus) of *Asparagus officinalis* measured using rabbit erythrocytes haemolysis assay are stated as percentage value. Ascorbic acid (1 mg/L) was used as the positive control. The data were analyzed by one-way ANOVA and the haemolysis percentage means of samples were compared using Duncan's Multiple Comparison Test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

4.2.3 DPPH* scavenging activity assay

Based on the DPPH* scavenging activity assay results (Figure 4.18), the highest scavenging capacity was obtained from *in vivo* plant extract (62.67%) followed by *in vitro* plant extract (61.33%) while there was no significant difference between these two samples. Callus extract (54.67%) showed significantly lower scavenging potential compared to the other plant extracts. Although all examined plant extracts showed significantly lower DPPH* scavenging activities compared to 1 mg/L of ascorbic acid (82.67%), all samples showed significantly higher free radical scavenging potential compared to 1 mg/L 2,6-di-t-butyl-4-methylphenol (BHT) (33.67%).

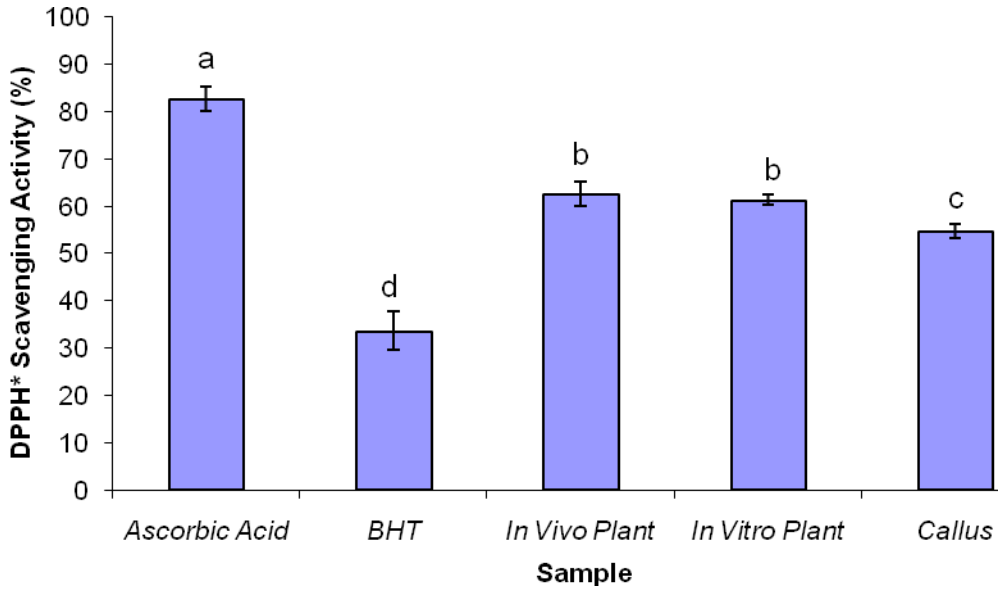

Figure 4.18: Antioxidant activity of examined plant extracts (*in vivo* plant, *in vitro* plant and callus) of *Asparagus officinalis* measured using DPPH* scavenging activity assay presented as percentage value. Ascorbic acid (1 mg/L) and BHT (1 mg/L) were applied as the positive controls. The data were analyzed by one-way ANOVA and the DPPH* scavenging activity percentage means of samples were compared using Duncan's Multiple Comparison Test (DMCT). Mean of different samples labeled with different letters are significantly different ($p < 0.05$).

4.3 Antibacterial activity assay

The results of antibacterial activity assay (Table 4.1) showed that bacterial growth inhibition was observed only in callus extract (100 mg/ml) against only one of the test pathogenic bacteria *Bacillus cerus*. The inhibition zone produced by callus (Figure 4.19) extract (14 mm) was significantly smaller than 30 µg tetracycline (40 mm). The rest of the extracts showed no antimicrobial activity in the same concentration of 100 mg/ml against any of the tested pathogenic bacteria.

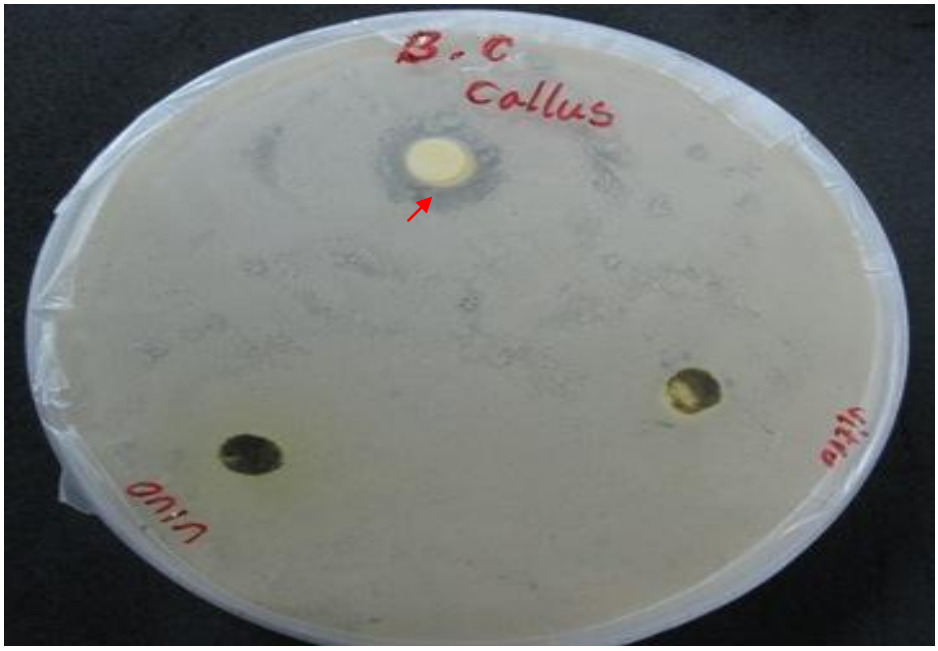

Figure 4.19: Comparison of antibacterial activity of *in vitro*, *in vivo* and callus extract of *Asparagus officinalis* in *Bacillus cerus*, using paper disk diffusion method

Table 4.1: Inhibition effect of 100 mg/ml of *Asparagus officinalis* ethanolic extracts (*in vivo* plant, *in vitro* plant and callus) against the growth of four pathogenic bacteria

Bacteria	**Inhibition Zone (mm)**			
	***In Vivo* Plant**	***In Vitro* Plant**	**Callus**	**Tetracycline (30 µg)**
Escherichia coli	-[b]	-[b]	-[b]	42 ± 3.00[a]
Staphylococcus aureus	-[b]	-[b]	-[b]	20 ± 2.64[a]
Pseudomonas aeruginosa	-[b]	-[b]	-[b]	11 ± 2.00[a]
Bacillus cereus	-[c]	-[c]	12 ± 1.00[b]	40 ± 3.60[a]

-: No inhibition; The data were analyzed by one-way ANOVA and the inhibition means of samples were compared using Duncan's Multiple Comparison Test (DMCT). Mean of different samples labeled with different letters are significantly different in each row ($p < 0.05$).