Results and Discussion
Plant regeneration through organogenesis can directly be achieved using different explants. The leaves of the *Curculigo orchioides* were found highly responsive when cultured. The explants responded for callus induction and direct organogenesis.

1. **Direct and Indirect organogenesis**

Regeneration of plantlets from leaf explants without intervening callus can be directly used for true-to-type traits in the propagules. Different media were tested for direct regeneration of shoots from the leaf segment by various researches (Suri *et al*, 2000; Prajapati *et al*, 2004; Thomas 2007). Suri *et al* (2000) observed large-scale multiplication of *Curculigo orchioides* from leaf explants in shake flask culture. The MS basal medium with reduced levels (½) of nitrogen (HH₄NO₃ and KNO₃) was found suitable for regeneration with various hormonal combination.

Earlier, the rhizome explants showed fungal contamination and exuded abundant phenolics in the medium (Prajapati *et al*, 2004). In present study, leaves were used as an explant for direct and indirect organogenesis. Leaf explants maintained on MS medium with ½
strength of nitrogen salts and supplemented with lower concentration of BA (0.44µM), developed shoots directly from the explant (Fig-6a). This is in accordance with the previous reports (Augustine and D'souza, 1997; Prajapati et al, 2004).

The first visible sign of shoot initiation was observed after 15 days, as white shiny protuberance on the explants (Fig-6b). The shoot buds enlarged in size and reached an average length of 9 cm in 12 weeks. It took about 12 weeks for complete organogenesis (Fig-6c). Rooting is an important step in the formation of complete plantlets. Auxins are considered to be the causative agents for root differentiation. The role of auxins in root development is well established (Scoot, 1972). In general, shoots are transferred to auxin containing medium, for root induction (Singh et al, 1981; Batra et al, 1991; Jain and Datta, 1992). However, according to Thorpe (1980) the endogenous auxin and cytokinin levels play an important role in root formation. Many researchers tried different media and growth regulators for root induction of Curculigo orchioides (Prajapati et al, 2003; Prajapati et al, 2004; Wala and Jasrai, 2003; Fransis et al, 2007; Thomas, 2007). The
higher level of BA in the medium inhibited the growth and shoot proliferation from the leaf explants (Suri et al, 1998).

BA contributes to organogenesis at lower concentrations, since higher concentrations have negative effect on shoot regeneration (Maity and Ghosh, 1997). Also, Lin and Chang (1998) have demonstrated that

Fig- 6: Shoot regeneration directly from leaf explants, a. leaf explant, b. shoot induction, c. root induction, d. complete plantlet (Horizontal bar = 1 cm)
higher level of cytokinins promoted shoot multiplication in *Bamboosa edulis* but elongation was restricted and vitrification occurred. Increased concentration of BA adversely affects the rate of shoot multiplication in *Thamnocalamus spathiflorus* and *Bambusa wamin* (Bag *et al*, 2000; Arshad *et al*, 2005). In fact, the leaf explant of *Curculigo orchioides* cultured on cytokinin-free medium produced entire plantlets from the cut ends of the leaf explants (Augustine and D’souza, 1997b). In present study, lower level of BA (0.44 µM) induced direct shoot organogenesis.

**Table- 5: Comparative study of number of shoots, roots, shoot length, root length, fresh and dry weight of generated plantlets*.**

<table>
<thead>
<tr>
<th>Organogenesis</th>
<th>Number of shoots</th>
<th>Shoot length</th>
<th>Root length</th>
<th>Fresh weight of plantlet</th>
<th>Dry weight of plantlet</th>
<th>Number of leaves</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(cm)</td>
<td>(mg)</td>
<td></td>
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</tr>
<tr>
<td>Direct</td>
<td><strong>10.3 ± 0.32</strong></td>
<td><strong>9.3 ± 0.4</strong></td>
<td><strong>2.3 ± 0.12</strong></td>
<td><strong>211.20 ± 19.5</strong></td>
<td><strong>19.8 ± 1.9</strong></td>
<td><strong>3.1 ± 0.08</strong></td>
</tr>
<tr>
<td>Indirect (Callus based)</td>
<td><strong>8.1 ± 0.7</strong></td>
<td><strong>7.03 ± 0.38</strong></td>
<td><strong>1.89 ± 0.15</strong></td>
<td><strong>222.716 ± 24.78</strong></td>
<td><strong>20.1 ± 1.3</strong></td>
<td><strong>3.75 ± 0.11</strong></td>
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</table>

*Mean ± SE of 70 replicates, data recorded after 12 weeks of inoculation.

In present study rooting was achieved on the same media. Plantlets obtained contained on an average of 3 leaves. The average length of shoots was 9 cm and length of roots was around 2 cm (Table-5).
Fig- 7: Shoot regeneration through callus, a. leaf explant, b. callus induction, c and d. well developed callus, e. shoot induction, f. complete plantlet (Horizontal bar = 1 cm)
Maximum 25 shoots per explants were obtained. Clusters having 1-5 shoots were robust and dark-green, having 4-8 leaves. Clusters of more than 5 shoots were comparatively weak and light-green with 2-4 leaves. The induction of plantlets directly from the parent tissue without intervening callus is of potential value for *in vitro* multiplication and storage of a given genotype. Direct organogenesis observed in this culture system forms an effective and rapid plantlet regeneration system.

The leaf explants maintained on MS medium with full strength nitrogen salts and supplemented with 2.22 µM BA, showed callus formation (Fig-7c). Thus normal level of nitrogen salts (as in basal medium) and higher amount of BA stimulated callus formation in *Curculigo orchioides*. Callus production in *Juniperus excelsa* on MS medium with half strength nitrogen was noted earlier (Shanjani, 2003). The present study revealed that callus induction in *Curculigo orchioides* was stimulated by full strength nitrogen salts of MS medium and higher level of BA. The higher concentration of BA induced callus formation from the cut ends of leaf explants in *Curculigo orchioides*
was also noted earlier (Augustine and D’sousa, 1997). Similar callus formation in *Citrus sinensis* with higher level of BA was noted (Moreira-Dias *et al*, 2000). Callus formation was initiated within 2 weeks from the cut ends of the leaf explants. High frequency of callus formation was obtained in next 15 days after initiation (Fig-7c and d). The callus was compact and pale-white with average fresh weight of 2.175 gm.

The callus further differentiated into globular structures which exhibits developed into shoot buds. The shoot buds ultimately led to the formation of shoots (Fig-7f). Though shoot and root formation in undifferentiated callus culture is dependent upon the specific equilibrium between auxin and cytokinin ratio, the shoot growth in *Curculigo orchioides* was observed on the medium containing cytokinin in absence of auxins. The exogenous requirement of the hormones depends on their endogenous levels in the plant system and varies with the tissue, plant type and the phase of plant (Batra *et al*, 2000).

The morphogenic compact callus showed shoot differentiation. These when transferred to MS medium with $\frac{1}{2}$ nitrogen salts and low level of
BA (0.44µM) produced shoots (Fig-7f). Shoots and roots developed within 3 weeks of the transfer. Data for average number of shoots, shoot length, root length, number of leaves, fresh weight and dry weight were recorded (Table-5), for generated shoots derived from indirect organogenesis (callus mediated). The average length of shoots obtained was 7 cm and length of roots was around 2 cm (Table-5).

Earlier researchers have tried different explants and growth regulators for the induction of direct and indirect organogenesis of *Curculigo orchioides*. Single shoot formation was achieved directly from leaf explant with BA (Augustine and D’souza, 1997b; Prajapati *et al*, 2003). Further, about 5 shoots were obtained using 2,4 D as growth regulator (Prajapati *et al*, 2003). Similarly, 4-5 shoot multiples (Wala and Jasrai, 2003) and 6-7 shoots (Fransis and Senapati, 2007; Sharma *et al*, 2007) directly from the shoot bud explants was reported earlier. Through indirect organogenesis, 4 shoots were obtained from leaf explants (Suri *et al*, 1998), 8 shoots from rhizome callus (Prajapati *et al*, 2003) and 11 shoots through stem discs (Nagesh *et al*, 2009). In the present study, almost 10 shoots per explant were derived from direct
organogenesis while 8 shoots were observed through indirect organogenesis (Table-5).

This is the first report on comparison of direct and indirect plantlet formation of *Curculigo orchioides*. Results showed that average number of shoots, shoot length and root length were higher in plantlets developed from direct organogenesis. In contrast, average fresh weight and dry weight of plantlets derived through callus was more than direct organogenesis (Table-5). The growth performance of regenerated plantlets (both through direct and indirect organogenesis) was evaluated separately.

The technique of *in vitro* regeneration of plants is of immense use as plants can be propagated throughout the year without dormancy and loss of quality. In contrast to the method, which ensures clonal stability, the regeneration of plant from callus may result in the genetic variability (Thomas, 1981) and loss of regeneration potential with age of culture (Murashige, 1984; Helperin, 1986). The callus cultures on periodic subculturing undergo genetic erosion, mutations and change in ploidy. Though highly undesirable for the maintenance of clones, the
main advantage of callus culture is the enormous number of plants which could be raised from a single culture, as every cell is a potential plant. Moreover, it ensures survival of the source plant in its natural habitat.

2. Shoots regeneration from upper/ middle/lower part of leaf Lamina

Explant factors such as its age, size and position etc are known to influence in vitro multiplication and growth (Botti et al, 1993; George, 1993). Part of the leaf used as an explant have an effect on the shoot multiplication rate and establishment of the cultures under in vitro conditions.

Correlation existing between the part of the leaf used as an explant on in vitro response, an experiment was carried out. Upper, middle and lower part of the leaf lamina were inoculated separately on MS medium with ½ strength of nitrogen salts and supplemented with lower concentration of BA (0.44µM). The shoots developed directly from the explants without any callus. As described in direct shoot organogenesis, both shoots and roots developed within 12 weeks. After 12 weeks the cultures were observed for average number of
leaves, number of shoots, shoot length, root length and fresh and dry weight of a plantlet.

Results clearly showed that the middle leaf lamina (MLL) gave best response in terms of number shoots. Average 10 shoots were emerged from middle leaf lamina (Table-6) while respectively 8 and 5 shoots were emerged from upper (ULL) and lower leaf lamina (LLL). The relative developmental age of leaf tissue used, dramatically influenced the shoot organogenic response observed for leaf explants (Brand and Lineberger, 1991). In *in vitro* cultures leaf part used as explant have an effect on the rate of *de novo* shoot formation of *Curculigo orchioides*.

Shoot formation from middle leaf lamina (MLL) were almost double in comparison with lower leaf lamina (LLL) but in other physiological parameters like, number of leaf, root length and fresh and dry weight of plantlet, lower lamina gave best response (Table-6). The average shoot length of plantlets from middle and lower leaf lamina were almost same (11 cm) while it was lesser in middle leaf lamina (9 cm).
Fig. 8: De novo shoots developed from a. upper, b. middle and c. lower leaf lamina (Horizontal bar = 1 cm)
Similar results were obtained in *in vitro* culture of apple. As regeneration capacity increased dramatically from the tip towards the base of the leaf, and was higher from the middle to the proximal end (Yepes and Aldwinckle, 1994). *De novo* shoots developed from ULL, MLL and LLL demonstrated on an average 3 leaves. It indicated that part of the leaf used as an explant have no effect on the number of generated leaves.

**Table- 6: Comparative study of number of shoots, roots, shoot length, root length, fresh and dry weight of generated plantlets from Upper, Middle and Lower leaf lamina**

<table>
<thead>
<tr>
<th>Leaf lamina</th>
<th>Number of shoots</th>
<th>Shoot length</th>
<th>Root length</th>
<th>Fresh weight of plantlet</th>
<th>Dry weight of plantlet</th>
<th>Number of leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPPER</td>
<td>8.0±0.25</td>
<td>8.9±0.4</td>
<td>2.4±0.16</td>
<td>189.0±18.76</td>
<td>18.8±2.06</td>
<td>3.1±1.85</td>
</tr>
<tr>
<td>MIDDLE</td>
<td>9.8±0.19</td>
<td>10.8±0.4</td>
<td>2.7±0.12</td>
<td>264.7±19.5</td>
<td>24.5±1.9</td>
<td>3.4±0.08</td>
</tr>
<tr>
<td>LOWER</td>
<td>5.0±0.12</td>
<td>11.2±0.65</td>
<td>4.3±0.66</td>
<td>347.2±39.21</td>
<td>35.1±4.71</td>
<td>3.7±0.15</td>
</tr>
</tbody>
</table>

*Mean ± SE of 50 replicates, data recorded after 12 weeks of inoculation.*

The growth in terms of average root length was higher (4 cm) in lower leaf lamina while it was almost same (2.5 cm) in shoots emerged from upper and middle leaf lamina (Table-6).
Biomass in terms of average fresh and dry weight was also studied. Results showed that average fresh and dry weight were higher in the plantlets raised from the LLL (Table-6). As the generated shoots were less in number in LLF and ULF, it must have resulted in more biomass of the plantlets while biomass was less in the plantlets of MLL because of the more number of generated shoots.

Regenerative capacity is known to increase substantially from the tip toward the base of the leaf. Differences in morphogenic capacity between the distal and proximal ends of leaves have been correlated with differences in maturity (Welander, 1988). The presence of a gradient of growth regulators and nutrients in the leaf may be responsible for the observed differences in organogenic ability along the explant, and for the increased regeneration observed in association with the vascular tissue (James et al, 1988; Welander 1988). Inherent tissue polarity can be nullified or altered by addition of growth regulators (Paterson, 1983). The complex effect of polarity on regeneration may involve both hormone transport and tissue maturity, since leaves mature from the tip to the base (Welander, 1988).
Orientation of the leaf explants on the medium has proven to be another important factor affecting the morphogenic response (Dufour, 1988; Welander, 1988). Regeneration is enhanced by placing the leaf segments abaxial side up on the medium, possibly due to increased oxygen exchange since stomata are located abaxially (Blancke and Belcher, 1989). This could also be due to the ability of palisade parenchyma on the adaxial surface to transport nutrients and growth regulators from the medium more efficiently into the explant (Welander, 1988).

In conclusion, among three different parts of the leaf used, the best response was achieved from middle part. It was used as an explant during all in vitro regeneration of *Curculigo orchioides*.

### 3. Histological analysis for de novo shoot formation

Middle lamina of the leaf explants gave significant morphogenetic response on MS medium supplemented with half-strength nitrogen salts and 0.44 µM BA. This protocol resulted in 100 % shoot regeneration response from inoculated explants.
Plant tissue culture system coupled with histology offer the possibility of identifying the point determination of cell differentiation leading to morphogenesis (Fukuda and Komamine, 1985). Thin sections of the leaf explants revealed origin of new structures from the cell initials associated with vascular bundles. Vasculature of this plant displays xylem to the adaxial side and phloem to the abaxial side with sclerenchymatous cells towards upper and lower epidermis respectively (Fig-9a). Histological examination of shoot formation process showed pro-meristemoids being formed in the sub-epidermal region in the midrib and veins. These structures developed subsequently into shoot primordia.

On MS medium with ½ strength nitrogen and low level of BA, the cultured explants exhibited cell cluster in the phloem parenchyma associated with vasculature in veins and midrib on 9th day of the culture (Fig-9c). The predominance of cell divisions in response to BA has been demonstrated earlier also (Sararitz et al, 1993; Villalobos et al, 1985; Von Arnold and Gronroos, 1986). The capacity of cell to respond to specific signals, probably of a hormonal nature, could lead
parenchymatous cell to dedifferentiate; subsequently forming a meristemoid and finally an organ (Thorpe, 1980).

Based on the cell division pattern and arrangement of the pro-meristemoid indicate likely origin from a single cell (Fig-9c). Within next 2-3 days, the parenchymatous cells exhibited periclinal divisions. In *Passiflora edulis* (Gloria *et al*., 1999) and *Fraser fir* (Saravitz *et al*., 1993) similar divisions were observed in the mesophyll cells close to the vasculature.

The continuous divisions in such cells lead to the formation of meristematic zone (Saravitz *et al*., 1993) consisting of small cells with dense cytoplasm. On day 15th of culture, with further cell divisions the vascular system seemed disorganized (Fig- 9d). Eventually, epidermal layers ruptured as the divisions progressed.

The first leaf primordia with meristemetic dome was observed on 22nd day (Fig- 9e), confirming that these structures originated from the cell located as sub-epidermal layers. The inception of new leaf primordial is indicated by the densely stained and well arranged cells.
Fig- 9: T.S. of leaf explants of *Curculigo orchioides* on a. 5th day, b. 7th day, c. 9th day, d. 15th day, e. 22nd day and f. 24th day of culture; DC = dividing cells; DV = disorganized vasculature; SP = shoot primordium (a, b, c and d 100x, c and d 40x)
Subsequent incubation of two days, leaf primordia was observed (Fig-9f) with cells arranged into longitudinal files covered by periclinaly devided elongated cells. A thick and continuous layer of elongated cells are likely to develop the epidermal cells of shoots.

The data clearly demonstrate that the shoots of *Curculigo orchioides* were developed from the meristemoids as a result of mitotic divisions in phloem parenchyma during first week of culture. Similarly in *Begonia erythrophyla* (Burritt and Leung, 1996) and conifers (Villalobos *et al*, 1985; Von Arnold and Gronroos, 1986; Saravitz *et al*, 1993) formation of shoots were reported directly from paranchymatous cells.

The histological studies with *Curculigo orchioides* clearly showed that plant regeneration occurred via *de novo* bud differentiation in 3 weeks of culture.

### 4. Subculture cycles

There are reports on *in vitro* multiplication via direct and callus mediated plant regeneration and embryogenesis from leaf explants. However, there is no report on the effect of subculture cycles on multiplication potential. The present study examined the impact of...
subculture cycles on multiplication of an important but endangered medicinal herb *Curculigo orchioides*.

Young leaves from *in vitro* raised plants of *Curculigo orchioides* (planted in 200ml thermocol cups) were used as an explant. Middle leaf lamina incubated on MS medium supplemented with half-strength nitrogen salts and 0.44 µM BA, resulted into direct shoot formation as described earlier (Fig-6). The first visible sign of shoot initiation was observed about 15 days of inoculation, as a white shiny protuberance, developed either from the midrib region or from cut ends of leaf explants. The growing shoots enlarged in size and reached an average length of about 10 cm in 12 weeks. However, no morphological variation was noted among the regenerated plantlets through subcultures.

In this study, maximum 10 shoots were obtained from first subculture (Fig-10 and 11). The earlier reports on shoot multiplication of *Curculigo orchioides* are quite varied. Augustine and D'suoza (1997b) and Prajapati *et al* (2003) reported formation of single shoot from a leaf explant using BA as growth regulator, while 5 shoots were
obtained using 2,4 D (Prajapati et al, 2003) and 8 shoots were obtained using BA+IBA+2,4,5-T (Thaker, 2004). Through indirect organogenesis, 4 shoots were noted using leaf explant (Suri et al, 1998). Wala and Jasrai (2003) reported 4-5 shoots from shoot apices using the MS media, while Fransis et al (2007) and Sharma et al (2007) reported 6-7 shoots using shoot bud as an explant.

**Fig- 10:** Effect of subculture cycles on shoot multiplication.*Mean+SE recorded after 12 weeks of growth

The present study clearly demonstrated 10 shoots (Fig-11a) in first subculture cycle. Moreover, the rate of shoot formation decreased gradually from first to seventh subculture cycle.
The shoot multiplication decreased gradually with increasing numbers of subculture cycles in banana (Jambhale et al, 2001; Lee, 2005). On the contrary, *in vitro* shoot formation rate steadily increased with each subculture cycle on hormonal medium in *Bacopa* (Sharma et al, 2010). While in *Gerbera*, multiplication began to increase starting from the 3rd subculture, reached its maximum in the 4th-6th subculture, and thereafter to declined (Vardza and Vardza, 2001). In present study, shoot formation decreased from 10 shoots in 1st subculture cycle to 4 shoots in 4th subculture cycle respectively (Fig-10).

![Fig-11: Shoots formation from different subculture cycles (SC), a. 1st SC, b. 7th SC, c. 12th SC. (Horizontal bar = 1 cm)](image-url)
5. RAPD analysis

One of the most crucial concerns in in vitro propagation is to retain genomic integrity of the micropropagated plants. There are reports indicating that the dedifferentiation of plant tissues for plant regeneration leads to genetic modifications (Hashmi et al. 1997, Rani et al. 2000). Williams et al (1990) developed Random Amplified Polymorphic DNA (RAPD) technology, which has since been widely used for the genetic analysis of biological systems. Somaclonal variation may result from genetic changes due to mutations or epigenetic changes or both (Smith, 1988). The frequency of variations has been found to increase with increase in sub-culture cycles (Chaterjee and Prakash, 1996). Moreover, critical variables for somaclonal variations also include genotype (Smith, 1988), source of explant (Vuylsteke et al, 1991; Martin et al, 2006), cultivation period and cultural conditions (Haisel et al, 2001).

The genetic fidelity of regenerated plants, derived through subculture cycles were assessed by RAPD analysis. Of the 20 primers tested (Table -7), OPE 04 and OPB 04 produced amplification products that were monomorphic across all the micropropagated plants (Fig-12). Primer
OPE 04 produced 5 bands and OPB 04 produced 3 bands common to all plants. These were consistently reproducible, well resolved fragments, in the size ranging from 500 bp to 1500 bp, when scored as the present or absent for RAPD markers (Fig-12).

Bands of equal molecular weight and mobility generated by the same primer can be considered to be of identical locus (Gillis et al, 2007). Intensity of bands at each locus depicting number of repetitive sequences was also found similar (Fig-11).

**Table- 7: Primers used for RAPD analysis**

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Primer</th>
<th>Sr No</th>
<th>Primer</th>
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<tbody>
<tr>
<td>1</td>
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<td>10</td>
<td>OPE 10</td>
<td>20</td>
<td>OPB 10</td>
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No variation was observed among the regenerants. This is more important, in light of the fact that each subculture cycle is of 10-12 weeks, thus some cultures were more than 70 weeks. Similar studies in
Vanilla plantifolia (Reddampalli et al, 2007) and Bambusa balcooa (Gillis et al, 2007) reported genetic uniformity among the micropropagated plants.

Fig - 12: RAPD profile of Curculigo orchioides regenerants with a. primer OPE 04, and b. OPB 04 from 1st to 7th subculture cycle, M indicates 1kb ladder.

Earlier uniformity in regenerated plants were noticed in Crotalaria longipes (Jayanthi and Mandal, 2001) and Arachis retusa (Gagliardi et al, 2004). Our results indicate that regenerated Curculigo plants from different subculture cycles were genetically alike and true-to-type.
6. Somatic embryogenesis

Somatic embryogenesis has a tremendous potential for large scale production of plant material and is considered as an effective aid in genetic transformation studies (Garcia and Martinez, 1995). It represents an alternative tool for massive clonal propagation. This appears to be a potential solution to the problem of field propagation, especially in area with frequent disease transmission and maintenance of cultivars that have been selected for important genetic characteristics (Amirato and Styer, 1985).

The leaf-age of explant plays a major role in somatic embryogenesis (Thomas and Jacob, 2004). Low frequency of embryogenesis and embryogenic induction related to the age of explant suggest that the intrinsic physiological stage of explant plays a decisive role in the induction of embryogenesis. Such observations were reported in somatic embryogenesis from zygotic embryo culture (Mathur, 2000; Gogate and Nadgauda, 2003). The middle lamina of the *in vitro* derived leaf explants (0.5 cm long) inoculated on MS media with 8µM and 15 µM BA showed callus formation. Callus formation was initiated within
2 weeks from the cut ends of the leaf explants (Thomas and Jacob, 2004).

High frequency of callus formation was obtained in next 15 days after initiation (Fig -13a). The callus was compact and pale-white in colour. Eventually, translucent patches of embryogenic cells were differentiated from the well developed callus.

On the MS medium with 8 µM BA, about 69 % cultures responded in terms of embryogenic calli, while about 40 % cultures developed embryogenic calli on MS media with 15 µM BA. Earlier, similar (89 %) response was obtained with 8 µM BA for direct embryogenesis (Thomas and Jacob, 2004). The translucent embryogenic calli when subcultured to the fresh medium (Dodeman et al, 1997) transformed into green embryoids (Fig-13b). The growth of embryoids was slow and occasionally get detached from the other cells and continued their growth. On an average 8 embryos were formed on MS medium with 8 µM BA while 4 embryos were found on MS medium with 15 µM BA. In fact, it is difficult to observe the different stages of embryogenesis in monocots (Thomas and Jacob, 2004).
Moreover, the heart shaped stage is absent in monocots as they have only one cotyledon (Sivakumar et al, 2003). The histological analysis of the embryogenic calli clearly showed globular shaped embryo (Fig-13c). When these embryoids were transferred to MS medium with $\frac{1}{2}$
strength nitrogen salts and supplemented with lower concentration of BA (0.44µM), developed shoots and roots (Augustine et al, 2008), giving rise to complete plantlets. Plantlets were then placed in thermocol cups containing mixture of soil, sand and compost (1:1:1; v/v/v) for hardening (Fig-13d).

7. Acclimatization

A substantial number of micropropagated plants do not survive transfer from in vitro conditions to greenhouse or field environment. The greenhouse and field have substantially low relative humidity, higher light level and septic environment that are stressful to micropropagated plants compared to in vitro conditions (Hazarika, 2005). The benefit of any micropropagation system can, however, only be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found ex vitro. Most species grown in vitro require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil (Hazarika, 2005).

Acclimatization has been defined as a controlled process, to adapt an organism to an environment change (Brainerd and Fuchgami, 1981). In
fact acclimatization is a crucial necessity since *in vitro* raised plants are not adapted for an *in vivo* conditions. *In vitro* raised plantlets of *Curculigo orchioides* were transferred to thermocol cups containing mixture of sand:soil:compost (1:2:2) and maintained under greenhouse condition (Fig-14a). Earlier, almost all the regenerated plants showed fungal infection and eventual wilting in non-autoclaved soil mixture (Wala and Jasrai, 2003). To avoid this, autoclaved soil mixture was used (Parmar and Jasrai, 2008). The plantlets were irrigated with ¼ strength MS medium. This helped plantlets to recover the shock of changes in its new environment. Earlier, Kar and Sen (1985) reported maintenance of plantlets on ½ strength of MS medium prior to their transfer to soil. For an initial period of about 2 weeks it is essential to maintain partial shade and humidity. Humidity could be maintained by spraying intermittent water mist. Similar process of maintaining humidity was practiced for hardening of Banana (Jasrai *et al*, 1999) and *Alpinia* (Rolf and Ricardo, 1995). The plantlets were gradually exposed to normal conditions by opening sides of poly-house after 2 weeks of incubation, the transferred to the plastic pots
containing mixture of sand:soil:compost (Fig-14c) followed by subsequent transfer in earthen pots (Fig-14d).

Fig 14: Acclimatized plantlets, a. in poly-house, b. in thermocol cups (2 year old plantlets), c. in plastic pots (2 year old plantlets), c. in earthen pot (4 year old plant).

The plantlets were allowed to grow at natural prevailing humidity and light intensity. On establishment the plantlets showed tremendous growth of rhizome, roots and lush green shoots without any morphological variation with 95 % survival. The acclimatized plants
flourished well (Fig-14d). They were evaluated for their performance in terms of fresh and dry weight of plants and size of rhizome. The experiment was carried out for four consecutive years.

8. Field Performance of *in vitro* regenerated plants

This study was conducted to evaluate the performance of *in vitro* derived plantlets of *Curculigo orchioides*. The pots were just maintained for the whole year and sprouted on onset of monsoon. Growth data were collected for 4 years including: number of roots, number of leaves, root length, shoot length, fresh weight of shoot and roots.

A gradual increase was observed every year in all the parameters studied, ie number of leaves, number of roots, root length, shoot length, root weight and shoot weight (Fig-15). Number of roots increased 1.1 %, 2.3 % and 2.5 % respectively in 2*nd*, 3*rd* and 4*th* year as compared to 1*st* year (Fig-16a). Similarly, number of leaves increased 2.15 % in the 4*th* year as compared to 1*st* year (Fig-16a). Root growth is very important as it has got immense medicinal value. Increase in root
weight observed was 1.47 %, 3.23 % and 10.0 % in 2nd, 3rd and 4th year while the root length increased to 1.55 %, 2.8 % and 4.4 % (Fig-16c).

Fig- 15: Growth performance evaluation of in vitro generated Curculigo orchioides, a. 1 year, b. 2 year, c. 3 year and d. 4 year old plant.
Fig- 16: Performance evaluation of *in vitro* generated *Curculigo orchioides*, a. Number of roots and leaves, b. Root and shoot length, c. Weight of shoot and root. *Mean ± SE* of data for recorded 25 plants.
Table 8: Element analysis of *in vitro* generated rhizomes of *Curculigo orchioides* and market sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe (ppm)</th>
<th>Cu (%)</th>
<th>Zn (%)</th>
<th>K (%)</th>
<th>Ca (%)</th>
<th>Na (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Market sample</td>
<td>2.46</td>
<td>0.18</td>
<td>0.33</td>
<td>2.12</td>
<td>0.70</td>
<td>0.85</td>
</tr>
<tr>
<td>1 yr old plant</td>
<td>2.27</td>
<td>0.16</td>
<td>0.52</td>
<td>5.06</td>
<td>0.05</td>
<td>1.12</td>
</tr>
<tr>
<td>2yr old plant</td>
<td>2.08</td>
<td>0.14</td>
<td>0.68</td>
<td>5.25</td>
<td>0.10</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Element analysis was performed to compare rhizome from market sample and *in vitro* raised plants. The results showed that amount of K and Zn were almost double in the *in vitro* raised rhizome than market sample. There was not much difference in the amount of other elements studied in the 1st and 2nd year. Earlier, the root tubers of *Curculigo orchioides* were investigated for its mineral content by Energy Dispersive X-Ray Spectroscopy (Agrhari *et al*, 2010). The EDX analysis showed that root tubers possesses only seven essential elements (C, O, Cl, K, Ca, Cu and Zn). The root tuber was found deficient in Mg, Al, Si and Fe earlier.

The growth pattern of *in vitro* raised plants were found to be normal indicating that *in vitro* raised plants were true-to-type and clonally
propagated. Field performance of tissue cultured plants were studied in number of species eg *Theobroma cacao* (Maximova *et al*, 2008), *Vaccinium vitis* (Gustavsson and Stanys, 2000), *Yucca valida* (Arce-Montoya *et al*, 2006), sugarcane (Sood *et al*, 2006) and banana (Msogoya *et al*, 2011). Rhizome production and total plant weight were also greater for the tissue cultured lignoberry in the field condition (Gustavsson and Stanys, 2000). Earlier, effectiveness of Arbuscular mycorhiza (AM) species on growth performance of *Curculigo orchioides* plants was reported (Sharma *et al*, 2008b; Sharma *et al*, 2009). The mycorrhizal plants were superior in most of the evaluated parameters, but the extent to which the growth of mycorrhizal plants was influenced varied with the inocula used. The plants inoculated with mixed consortia containing maximum AMF species richness exhibited improved growth in comparison to consortia containing lower AMF diversity and monospecies cultures (Sharma *et al*, 2009).
9. HPTLC

In the past few years, high performance thin layer chromatography (HPTLC) has emerged as a potential tool for rapid and useful phytochemical evaluation of herbal drugs (Shah et al., 2000). HPTLC analysis of medicinal plants is important for quality control and batch-to-batch reproducibility of botanical products (Sharma and Patel, 2009). The phytochemical constituents in a plant material form a characteristic fingerprint, representing quantity of the active constituents. Moreover, this helps to standardize the mixture unlike formulated herbal drug and market samples (Paramasivam et al., 2008).

Different solvent systems of varied polarities are used to resolute components present in the fractions (Bafna and Mishra, 2005). In the present study, HPTLC profiles of market samples (rhizome powder and pieces of rhizome), rhizome and leaves of in vitro raised plantlets and ex vitro rhizomes of *Curculigo orchioides* were developed. Chromatogram of *Curculigo orchioides* developed in ethyl acetate: methanol: water (10:1.35:1; v/v/v) exhibited maximum number of bands in methanol extract in UV and visible range. *In vitro* raised
rhizome and market product exhibited maximum number of bands at 366 nm. The lowest numbers of bands (8) were found for *in vitro* leaves at 254 nm under visible light (Fig-17).

**Fig- 17: HPTLC chromatogram visualized in various wavelength representing different compounds**

**Fig- 18: Densitometric chromatogram showing peak display**

Earlier, curculigoside was extracted in methanol from the crude medicine through Sep-Pak C 18 cartridges based purification and
Curculigoside were detected at UV 283 nm by ODS-3 chromatographic column using methanol-water-acetic acid (45:80:1) as a solvent system. The content range of the curculigoside for 6 kinds of different samples was from 0.11 % to 0.35 %.

Similarly, orcinol glucoside, orcinol-1-O-β-D-apiofuranosyl-(1-O-β)-D-glucopyranoside, was isolated by column chromatography from the rhizomes of *Curculigo orchioides* (Wu et al, 2005). Phytochemical investigations on *Curculigo orchioides* revealed the presence of a novel pentacyclic triterpenoid (Mehta and Gawarikar, 1991). Four phenol glycosides have been identified as curculigoside, orcinol glucoside, curculigine A and corchioside (Kubo et al, 1983; Garg et al, 1989). Similarly, cycloartane type glycosides and their glycosides were also reported (Xu and Xu, 1992a). Recent studies showed that a number of plants products include polyphenolic substances such as flavonoids and tannins. These natural antioxidative substances usually have a phenolic moiety in their molecular structure. They have been found among flavonoids, tocopherols and catechines.

Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or have synergistic effects when used together with
phenolic antioxidants. The activity of methanol extract of *Curculigo orchioides* may be due to presence of phenolic glucosides (Bafna and Mishra, 2005).

**10.0. Impact of elevated CO₂ on *in vitro* multiplication of *Curculigo orchioides***

Carbon dioxide and light are important for growth and development of plantlets *in vitro* (During and Harst, 1996). The cultures were grown on BA (0.44 µM) supplemented MS medium with or without sucrose (2%). A range of CO₂ concentrations in glass-chamber was used for *In vitro* growth and multiplication of *Curculigo orchioides*. For CO₂-free environment, a saturated solution of KOH was kept in the chambers (Vyas and Purohit, 2003). The comparative study of various growth parameters like number of shoots, shoot length, root length, fresh and dry mass of shoots produced, number of leaves per plant, leaf area and chlorophyll content were followed with elevated (continuous/intermittent) CO₂ supply.
10.1. Impact of elevated CO$_2$ (with and without sucrose) shoot regeneration and growth of *Curculigo orchioides*

Miropropagation can also be carried out in sugar-free medium, ie photoautotrophic condition, using elevated CO$_2$ as a carbon-source. This approach has been shown to reduce the risk of microbial contamination (Kozai, 1991; Pospisilova *et al*, 1999; Deng and Donnelly, 1993; Yoon *et al*, 2009). But in the present study, when CO$_2$ was given as sole carbon source (sucrose free medium) leaf explants sustained for only 7-8 days with subsequent death. Similar results were obtained in photoautotrophic cultures of tobacco (Solarova *et al*, 1989; Ticha, 1996), *Wrightia tomentosa* (Vyas and Purohit, 2003) and *Chlophophytum borivilianum* (Joshi *et al*, 2009).

The plantlets could not survive and subsequently deteriorated in the sucrose free media (Solarova and Pospilova, 1997). While, Potato plantlets responded in sugar-free media although sugar containing media performed better (Rahman and Alsadon, 2007). Shoot cultures of *Achras zapota* grown on sucrose containing medium under CO$_2$ free condition showed significant decline in all the growth parameters.
Results clearly showed that shoot clusters derived from leaf explant produced more than five shoots in ambient air (0.36 % CO₂) of growth room after 12 weeks of growth (Fig-19). Fresh weight of the shoots was about ten times higher than the dry mass (Fig-19c). In comparison to ambient air control, the cultures grown under CO₂ free atmosphere showed significant decline in all the parameter studied (Fig-19a, b and c). In the ambient air of the growth room, cultures were characterized by thin shoots, with lesser number of leaves, reduced leaf area, and reduced fresh and dry masses.

At 1.0 % CO₂, improvement in shoot regeneration and growth was observed. Under these conditions significant increase in number of shoots per explant, fresh and dry masses and leaf number over the absence of CO₂ was obtained (Fig-19a, b and c). Under CO₂ free condition shoots exhibited very poor growth in all parameters studied. Maximum number of shoots were obtained at 1.0 % but the growth in terms of shoot length, root length and fresh and dry weight of shoots was maximum at 2.5 % CO₂ level.
Fig-19: Effect of CO₂ enrichment on growth parameters of *in vitro* cultured *Curculigo orchidoides*, a. Number of shoots and leaves, b. Shoot and root length (cm), c. Fresh and dry weight (mg), d. leaf area (cm²) and Chlorophyll content (mg/gm) *Mean ± SE of 10 replicates after 12 weeks of growth.*
The chlorophyll content of plants plays an important role in photosynthesis. The chlorophyll content of plantlets was significantly influenced by the levels of applied CO₂ (Fig-19d). The chlorophyll content increases with increased level of CO₂. Among all the treatments, the chlorophyll content was greatest in 2.5 % CO₂ (Fig-19d). This demonstrates that CO₂ could improve photosynthetic ability of plantlets by stimulating chlorophyll synthesis and thus in turn improves growth of plantlets (Zhang et al, 2009).

At 2.5 % CO₂, cultures did not show any improvement in shoot proliferation but quality of cultures in terms of shoot length, root length, fresh and dry mass was better in comparison to 0.1 % CO₂ concentration (Fig-19 a, b and c).

Results demonstrated a marginal increase in leaf area at elevated level of CO₂ from 0.5 % to 2.5 % CO₂ (Fig-19d). Similar results have been obtained in case of Wrightia tomentosa (Vyas and Purohit, 2003). Leaf area was found to be greater with elevated CO₂ for a number of plant species, including Musa (Schaffer et al, 2006), avocado (de la Vina et al, 1999) and wheat (Fusheng et al, 2004). On the contrary, Ewert (2004) reported that elevated CO₂ has little or no effect on leaf area index to
control growth responses and presumed that it was largely affected by radiation saturation. Moreover, relationships between plant growth, elevated CO$_2$ and leaf area index are not well understood (Ewert, 2004).

Fresh and dry weight of shoots increased with increasing levels of CO$_2$ (Fig-19c). Earlier, dry matter accumulation was found to increase with enhanced levels of CO$_2$ during a number of _in vitro_ studies (Ferris and Taylor, 1993). Shoot biomass was enhanced in elevated CO$_2$ for _Sanguisorba minor_ and _Lotus carniculatus_, while there was no effect of elevated CO$_2$ on shoot biomass for _Anthyllis vulneraria_ (Ferris and Taylor, 1993).

Kozai and Iwanami (1988) have explained the comparatively poor growth under ambient CO$_2$, to be due to decline in CO$_2$ concentration inside the culture vessel during most of the photoperiod. In controlled environment the continuous availability of CO$_2$ compensate for the depletion of its concentration and therefore, encourage better shoot growth as compared to ambient air control (Solárová _et al_, 1989, Jeong _et al_, 1995). However, it was suggested that in C4 plants, CO$_2$-enriched air reduces photosynthesis unlike C3 plants (Erickson _et al_, 2006).
Results and Discussion

Photosynthetic capacity of many plant species grown under elevated CO₂ was shown to get reduced by a variety of factors including limited sink capacity, nitrate limitation, end-product limitation, excess accumulation of starch, a decrease in photosynthetic enzymes such as Rubisco, and accelerated senescence (Sun et al., 2002). It was suggested that when output exceeds the plant’s capacity to metabolize or export sugars, increasing carbon-metabolites repress the photosynthetic and/or metabolic genes (Jaime, 2004).

10.2. Impact of elevated CO₂ (intermittent and continuous CO₂) shoot regeneration and growth of Curculigo orchioides

Results of previous experiment revealed that leaf explants of Curculigo orchioides could not survive in absence of sucrose. Therefor along with elevated CO₂ sucrose was provided in the medium. Experiment with elevated CO₂ was conducted for the continuous (CCS) and intermittent (ICS, 7 hr) CO₂ supply.

Shoots regenerated under controlled CO₂ environments showed varied responses in both CCS and ICS. Under CCS ambient air control set produced about 4 shoots per explant (Fig-22a). Under intermittent CO₂
supply (ICS), ambient air (0.36 %) produced about 3 shoots per explant with total fresh weight more than 9 times than total dry weight (Fig-23c), indicating nearly 2.8 cm² leaf area and 4.4 mg/gm chlorophyll content (Fig-23d). In ambient air (0.36 % CO₂) of the growth room, cultures grown under ICS and CCS showed decline in all the parameters recorded as compared to CO₂ enriched condition. The natural CO₂ concentration in the growth room is not sufficient for optimal shoot growth and multiplication (Kozai et al., 1992). Regardless of the sucrose content of the medium, CO₂ enrichment has been reported to be effective for promoting the growth of Cymbidium, Carnation and Potato (Kozai et al., 1992). In the woody plants CO₂ enrichment was effective for promoting growth of Pinus radiata (Aitken-christie et al., 1990). Declined shoot growth under ambient air was recorded in number of plants eg Rhododendron (Aracama et al., 2001), Phalaenopsis (Yoon et al., 2009), Epidendrum (Giang and Tanaka, 2004), Achras zapota (Dave and Purohit, 2004) and Wrightia tomentosa (Vyas and purohit, 2003).
Leaf area
The leaf area did not show remarkable change under CCS (Fig-22d) while leaf area increased by elevated CO$_2$ under ICC (Fig-23d). However the best response was obtained in 2.0 % CO$_2$ under CCS and 2.5 % CO$_2$ under ICS. A positive influence of greater leaf area on the growth rate under autotrophic conditions has been reported previously (Kozai et al, 1992). Leaf expansion and production were stimulated by elevated CO$_2$ for _Sanguisorba minor_, _Lotus corniculatus_ and Plantago, while for _Anthyllis vulneraria_, production of broader type as compared to those produced in ambient CO$_2$. Contrasting effects of CO$_2$ were also apparent for measurements of specific leaf area, which increased for _Lotus carniculatus_, decreased for _Anthyllis vulneraria_ and remained unaltered for _Sanguisorba minor_, and Plantago media in elevated compared to ambient CO$_2$ (Ferris and Taylor, 1993).

Chlorophyll content
The chlorophyll content plays an important role in photosynthesis. The chlorophyll content was influenced by the different level of applied CO$_2$. Chlorophyll content increased under CO$_2$ enriched conditions during ICS (Fig-23d). Among all the treatments, the chlorophyll
content was greatest in 2.5 % CO₂ (Fig-23d). This demonstrates that CO₂ could improve photosynthetic ability of plantlets by stimulating chlorophyll synthesis and thus in turn improves growth (Zhang et al, 2009).

There was not continuous increase/decrease with the increasing CO₂ under CCS. The effect of elevated CO₂ on chlorophyll content has been found to vary with the plant species. Similar results were obtained in potato. Elevated CO₂ had no significant effect on chlorophyll content in Potato (Donnelly et al, 2001). Chlorophyll concentration was unaffected by elevated CO₂ (Carswell et al, 2000). Elevated CO₂ reduced the Chl a+b and Chl a (Zhao, 2010). This is consistent with Luomala et al (2003) who reported that elevated CO₂ reduced the chlorophyll content. Uprety and Mahalaxmi (2000) attributed such a reduction to the reduction in Rubisco.
Fig-20: *In vitro* regeneration of *Curculigo orchioides* under elevated CO₂ (continuous) supply. a. 0.0 %, b. 0.36 %, c. 0.5 %, d. 1.0 %, e. 2.0 %, f. 2.5 %, g. control(c) and h. CO₂ chamber (Horizontal bar=1cm).
Fig-21: *In vitro* regeneration of *Curculigo orchioides* under elevated CO$_2$ (intermittent) supply a. 0.0 %, b. 0.36 %, c. 0.5 %, d. 1.0 %, e. 2.0 %, f. 2.5 %, g. control(c) and h. CO$_2$ chamber (Horizontal bar=1cm).
Fig -22: Effect of CO₂ enrichment (continuous) on growth parameters of in vitro cultured Curculigo orchiodies, a. Number of shoots and leaves, b. Shoot and root length (cm), c. Fresh and dry weight (mg), d. leaf area (cm²) and Chlorophyll content (mg/gm) *Mean ± SE of 4 experiments with 10 replicates each, after 12 weeks of growth.
Fig -23: Effect of CO₂ enrichment (Intermittent) on growth parameters of *in vitro* cultured *Curculigo orchiodies*, a. Number of shoots and leaves, b. Shoot and root length (cm), c. Fresh and dry weight (mg), d. leaf area (cm²) and Chlorophyll content (mg/gm) *Mean ± SE of 2 experiments with 10 replicates each, after 12 weeks of growth.*
Fig-24: Impact of elevated CO₂ (continuous) on micro-elements, a. Fe (ppm), b. Cu (ppm) and c. Zn (ppm)*Mean ± SE of 4 experiment with 10 replicates in each, after 12 weeks of growth.
Fig-25: Impact of elevated CO$_2$ (continuous) on macro-elements, a. K (%), b. Ca (%) and c. Na (%). *Mean ± SE of 4 experiment with 10 replicates in each, after 12 weeks of growth.
Fig-26: Impact of elevated CO$_2$ (intermittent) on micro-elements, a. Fe (ppm), b. Cu (ppm) and c. Zn (ppm) *Mean ± SE of 4 experiment with 10 replicates in each, after 12 weeks of growth.
Fig-27: Impact of elevated CO₂ (intermittent) on macro-elements, a. K (%), b. Ca (%) and c. Na (%) *Mean ± SE of 4 experiment with 10 replicates in each, after 12 weeks of growth.
In *Phalaenopsis* plantlets, chlorophyll content was enhanced during *in vitro* CO₂ enrichment at the concentration 1.0% (Yoon *et al.*, 2009). In contrast, elevated CO₂ caused a faster decline in chlorophyll content in spring wheat cv Minaret (Ommen *et al.*, 1999) and in Carob tree (Osorio *et al.*, 2005) as compared to ambient condition. However, chlorophyll a/b ratio was significantly higher in the leaves of Carob tree under elevated CO₂ condition. Under photomixotrophic conditions, no constant increase or decrease was maintained in total chlorophyll content in *Curculigo orchioides* when leaves of both elevated and ambient conditions were compared under CCS.

**Element Analysis**

Results with continuous and intermittent CO₂ supply, clearly showed that the levels of macro-elements (K, Ca and Na) are more amount in leaves while the micro-elements are maximum in roots (Fig-24 to 27). In contrast to this study, microelements were recorded higher than macro-elements in leaves while the macro elements were obtained trace amount in roots of rice (Lieffering *et al.*, 2004). Earlier, the root tubers of *Curculigo orchioides* were investigated for its mineral content
(Agrhari et al, 2010). The root tubers were noted to possess C, O, Cl, K, Ca, Cu and Zn and deficient in Mg, Al, Si and Fe earlier.

Results showed that the amount of elements was not influenced by the elevated CO$_2$ (Fig-24 to 27). There was not continuous increase or decreases in the selected elements under ICS and CCS. Similar results were obtained in rice grown under elevated CO$_2$. No changes were found in the concentration of macro and micro-elements analyzed in rice (Lieffering et al, 2004). In contrast to our study elevated CO$_2$ decreases the amount of nutrient element in number of plants (Cotrufo et al, 1998; Gifford et al, 2000; Fangmeier et al, 2002). Under elevated CO$_2$ all the micro and macro nutrient elements (N, K, P, Ca, Ma, Mn Zn Fe) tended to decrease in potato (Fangmeier et al, 2002). Loladze (2002) observed the reduction in the concentration of elements in wheat gain under elevated CO$_2$. As CO$_2$ concentrations increase, plants typically show increased concentrations of carbon in their tissues, with correspondingly reduced concentrations of other elements, including nitrogen (Cotrufo et al, 1998; Gifford et al, 2000), phosphorus (Gifford et al, 2000) and several trace elements (Loladze, 2002).
Shoot regeneration and growth
Elevated CO₂ greatly influence the regeneration of shoots. Under enriched environment by different concentrations of CO₂ (0.36-2.5 %), the shoots showed an improved growth over shoots grown under ambient air in both CCS and ICS (Fig-22 and 23). Number of shoot increased with increasing CO₂ levels. The best response in terms of shoots number was obtained at 2.5 % CO₂ in ICS and CCS.
Similar results were obtained in photomixotrophic culture of Chlorophytum borivianum (Joshi et al, 2009). In Chlorophytum borivianum, 2.5 % CO₂ enhances shoot multiplication. However, in tobacco (Solarova and popisilova, 1997), Carnation (Solarova and popisilova, 1997), Wrightia tomentosa (Vyas and Purohit, 2003) and Achras zapota (Dave and Purohit, 2004) the best response was obtained with 0.5 % CO₂ supply. At 2.5 % CO₂, about 9 shoots were obtained in CCS while 7 shoots were obtained under ICS in Curculigo orchioides (Fig-22a and Fig-23a). At 2.5 % CO₂, almost 2 fold shoots were produced as compared to control under CCS and ICS (Fig-21a and Fig-22a).
Shoot and root length of plantlets produced in each treatment had marginal difference under CCS (Fig-22b), however shoot and root length was increased with increasing levels of CO₂ under ICS (Fig-23b). Similarly, exposure to elevated CO₂ promoted root extension in *Sanguisorba minor*, *Lotus corniculatus* and *Plantago* but root length was not promoted by elevated CO₂ in *Anthyllis vulneraria* (Ferris and Taylor, 1993). Specific root length was more in elevated CO₂ for *Plantago* while the root to shoot ratio remained unchanged by CO₂ (Ferris and Taylor, 1993).

The shoot biomass in terms of fresh and dry weight was better under CO₂ enriched conditions than control. But there was not constant increase in fresh and dry weight at different CO₂ levels in *Curculigo orchioides*. Similarly, elevated CO₂ has been shown to increase biomass production in *in vitro* multiplication of *Momordica grosvenori* (Zhang *et al*, 2009), *Sanguisorba minor*, and *Lotus corniculatus*, whilst there was no effect of elevated CO₂ on shoot biomass for *Anthyllis vulneraria* (Ferris and Taylor, 1993).

Results showed that in comparison to ICS, CCS proved better in terms of shoot numbers and shoot biomass (fresh and dry weight) while
there was a marginal increase observed in shoot and root length under ICS than CCS (Fig-22a and c; Fig-23a and c). Number of leaves, leaf area and chlorophyll content was not affected by the duration of CO$_2$ supply (continuous and intermittent). However under both treatment (ICS and CCS) the best response in terms of shoot numbers, shoot length fresh weight was obtained at 2.5 % CO$_2$ level.

Results of present study revealed that photoautotrophic (sugar free media) growth of *Curculigo orchioides* could not be possible as the explant could not survive with CO$_2$ supplied as sole carbon source. Leaf explants deteriorate and died in absence of sucrose. The photomixotrophic growth of *Curculigo orchioides* could be achieved when both sucrose and CO$_2$ were provided. Regardless of the sucrose content of the medium, CO$_2$ enrichment has been reported to be effective in promoting growth of Cymbidium (Kozai *et al*, 1987), *Dianthus* (Kozai and Iwanami, 1988), *Fragaria* (Fujiwara *et al*, 1988), *Solanum* (Kozai *et al*, 1988) and *Nicotiana* (Solarova *et al*, 1989).

The combination of sucrose (2.0 %) and CO$_2$ (2.5 %) proved to be the best for shoot multiplication where maximum
number of shoots were obtained during the present study. Similar results were obtained in Carnation (Solarova and Pospisilova, 1997), tobacco (Ticha, 1996), apple (Morini and Melai, 2003), *Feronia limonia* (Vyas and Purohit, 2006) and *Chlorophytum borivilianum* (Joshi *et al*, 2009). The growth of shoots in terms of number of shoots, shoot length, root length, number of leaves, leaf area and chlorophyll content was better under CO₂ enriched (2.5 %) conditions rather than ambient air. Kozai and Iwanami (1988) have explained the comparatively poor growth under ambient CO₂ to be due to decline in CO₂ concentration inside the culture vessel during most of the light period (Pospisilova *et al*, 1992; Gabriela *et al*, 2005). In controlled environment, the continuous availability of CO₂ perhaps compensated for the depletion of its concentration and, therefore, encouraged better shoot growth as compared to ambient air control (Solarova *et al*, 1989; Jeong *et al*, 1993; Zoyabed *et al*, 2000).

Nowak and Shulaev (1986) have observed that photoautotrophic/ photomixotrophic mode suit well for the micropropogation, which might prove useful for the successful
hardening and acclimatization of tissue cultured plants. The technique may be economically feasible for the large scale production. Newly developed photoautotrophic micropropagation systems were adapted to produce quality plant propagules at minimum usage of resources, to maximize production and minimize cost (Rahman and Alsadon, 2007).

Kubota and Kozai (1992) successfully developed the photoautotrophic growth of potato plantlets with forced ventilation. Further, photoautotrophically produced propagules do not require acclimatization prior to transplanting in the *ex vitro* conditions (Kozai *et al*, 1987, Fujiwara *et al*, 1995). This system can be used for different horticultural and woody plant species with minimum modification (Chun and Kozai, 2001). Hayashi *et al* (1995) reported that the number of air exchange and lighting cycle are important for the photoautotrophic/photomixotrophic growth of plantlets. Air-tight culture vessel showed lower performance than those of cotton plug capped vessels due to lack of proper ventilation. Extreme fluctuations of CO₂ concentrations between duration of light and darkness cycles can occur in air tight vessel but those with natural ventilation or with forced ventilation (Zoyabed *et al*, 1999). Autotrophic/mixotrophic
micropropagation can improve growth and development of multiplying cultures. It has an advantage over conventional micropropagation (Jeong et al, 1995).