DISCUSSION
5.1. Micropropagation of *C. orchioides*

Explant preparation, particularly surface disinfection is generally considered as one of the most important steps for introduction of plant material into *in vitro* culture without any microbial contaminants (Teng *et al*., 2002). Standardization of disinfection procedure is a critical step for successful establishment of aseptic culture. In our study, high incidence of microbial contamination was found to be often responsible for poor establishment of aseptic culture of leaf and rhizome explants. Rhizome explants of *C. orchioides* when disinfected with HgCl$_2$ (0.1 %) and NaOCl (1 %) for 15 min, maximum of about 5.17 and 12.1 explants could be recovered out of 30 explants from the respective treatments without any visible microbial contamination. In the present study, disinfection of leaf explants with HgCl$_2$ (0.1 %) for 6 min shown the highest mean number of explant recovery (16.2). Whereas, disinfection of leaf explants with NaOCl (1 %) for 15 min yielded an average of only 13.4 explants out of 30 explants. In general HgCl$_2$ was reported to be an effective disinfectant and widely used in micropropagation of several medicinal plants such as *Curcuma zedoaria* (Stanly and Keng, 2007), *C. orchioides* (Nagesh, 2008), *Thalictrum dalzellii* (Sharanappa and Rai, 2011). Thus, the present study revealed that HgCl$_2$ is more effective than NaOCl for elimination of microbial contamination in rhizome and leaf explants of *C. orchioides* as reported in many plant species.

The loss of explants due to phenolic oxidation is considered to be one of the major problems in micropropagation of medicinal plants. Phenolics are secondary metabolites that modulate plant development (Arnaldos *et al*., 2001) and protect plants against various diseases and pests (Kefeli *et al*., 2003; Conceicao *et al*., 2006; Fan *et al*., 2006). However, many authors have reported that phenolics also generate toxic compounds and have inhibitory effect during *in vitro* regeneration of several trees (Toth *et al*., 1994; Laukkanen *et al*., 1999; Dibax *et al*., 2005) and crop species (Bieri *et al*., 1984; Selva *et al*., 1989; Prajapati *et al*., 2003; Sharada *et al*., 2003). When explants are injured, they exude phenolic compounds that readily oxidized and inhibit the activities of several enzymes. As a result, the explants often turn into brown, leading to
darkening or browning of culture medium as reported by many authors (Compton and Preece, 1986; Bhatt and Chandel, 1991; Lainé and David, 1994; Laukkanen et al., 1999; Arnaldos et al., 2001).

In the present investigation, freshly cultured rhizome explants often exuded large quantity of phenolic compounds, leading to loss of considerable number of explants during initiation of cultures. In order to overcome this problem, systematic experiments were carried out. Rhizome and leaf explants cultured on MS medium supplemented with activated charcoal (3 g/l) along with 4.4 µM BAP shown higher recovery in both rhizome and leaf explants. Lowering the concentration of charcoal (1 - 2 g/l) in the medium reduced the overall recovery of the cultures in both the types of explant. Presence of activated charcoal (3 g/l) in the medium during initial culture period did not inhibit the bud break and shoot regeneration in rhizome as well as in leaf explant. In this experiment, both rhizome and leaf explant shown 63.8 % and 48 % recovery respectively. The beneficial effect of activated charcoal during in vitro culture of many higher plants was emphasized (George, 1999).

It was also observed that freshly cultured rhizome and leaf explants showed bacterial and fungal contamination within a week of culture and most of the explants were found to leach phenolic compounds into the medium. Explants cultured in absence of charcoal revealed high rate of browning and death of explants within two weeks of culture. In contrast, presence of activated charcoal in the medium improved the viability of explants even after 30 days of culture. This could be possibly due to rapid adsorption of toxic compounds (oxidized products of phenols) by the charcoal to protect the exposed tissues for better survival and regeneration. In support of these findings, beneficial effect of activated charcoal on early response and elongation of microshoots was reported in many plants such as Jovellana punctata (Ronse, 1995), Juglans regia L (Saadat and Hennerty, 2001) and Taxus wallichiana (Datta et al., 2006). It was reported that addition of activated charcoal in the culture medium prevented the inhibitory effect of phenolics on growth and development of Celastrus
paniculatus and Curculigo orchioides (Sharada et al., 2003; Prajapati et al., 2003). Furthermore, it was also reported that use of activated charcoal in the medium enhanced the shoot elongation. In addition, the shoots cultured in presence of activated charcoal had produced dark green leaves as compared to control during in vitro culture of Eucalyptus (Dibax et al., 2005).

PVP was frequently used in control of browning during in vitro culture of several woody species. However, browning of rhizome and leaf explants of C. orchioides could not be controlled by using PVP. Various concentrations of PVP (0.44, 0.66, 0.88 and 1.77 mM) were tested to overcome the problem of browning in rhizome and leaf explants of C. orchioides. Nevertheless, no effective concentrations could be identified. In contrast to this finding, presence of 1 g/l PVP in the culture medium reduced the secretion of phenolic substance in stem segment of Aloe vera (Roy and Sarkar, 1991). Polyvinylpyrrolidone (PVP) was very often used to reduce the secretion of phenolic substances from the explants of tree species. Studies carried out with several woody plants had suggested that presence of PVP in the media reduce the browning in cultured tissue (Gupta et al., 1980; Tyagi et al., 1981; Zhong et al., 1995). Micropropagation of C. orchioides was attempted previously by Prajapati et al., (2003). In this study, PVP was reported to be ineffective in control of browning as observed in the present study.

Use of antioxidants and adsorbents were found effective in controlling the browning of explants in many species such as Cattleya sps (Ichihashi and Kako, 1977), C. zeylanicum (Rai and Jagadishchandra, 1987), walnut (Rodriguez, 1982), Rhododendron (Preil and Engelhardt, 1977) and several other species. The positive effect of different antioxidants and adsorbents could be due to specificity of these chemicals only to certain plant species. In support of this fact, it was established that PVP is effective in controlling browning only in selected species (Vaugh and Duke, 1984). Thus, the ineffectiveness of PVP in control of browning in rhizome and leaf explants of C. orchioides could be attributed to specificity of this compound only to certain species.
In the present study, incubation of rhizome and leaf explants under dark condition shown positive effect in control of browning as revealed by the maximum recovery of explants in rhizome (9.08 ± 1.24) and leaf (12.58 ± 0.90). But in control, the response of explant was significantly reduced in rhizome and leaf explants. The beneficial effect of dark incubation to overcome the problem of browning in different explants of several species was reported (Scalbert et al., 1990; Dodds and Roberts, 1995; Khatri et al., 1997; Zeweldu and Ludders, 1998; Abeyaratne and Lathiff, 2002; Titov et al., 2006). Although, various methods were employed for overcoming the browning, incubation of cultures under dark for the initial culture period was reported to be effective (Bajaj, 1977; Durand-Cresswell and Nitsch, 1977; Birmeta and Welander, 2004; Titov et al., 2006; Kiong et al., 2007). Maintenance of cultures in dark suppresses the metabolic activities since the product of phenolic oxidation are formed under light condition (Chawla, 2002). In different plant species such as Gladiolus (Ziv et al., 1970), Euretrentricosum (Birmeta and Welander, 2004) and Strelitzia reginae (Strosse et al., 2009), incubation of culture under dark was reported to be effective in reducing the browning of explants in micropropagation.

Despite the large number of reports on micropropagation of several plant species, loss of explants due to phenolic exudation is still considered as a major problem in micropropagation and *C. orchioides* has no exception. In the present study, different experiments were carried out to overcome the harmful effect of phenolic oxidation. Experiment carried out with ascorbic acid, citric acid and L-Cysteine-HCl for controlling the browning of rhizome and leaf explants of *C. orchioides* revealed positive results. This experiment could also lead to identification of selective antioxidants with optimal concentrations for control of browning. Of the various treatments, the optimum concentrations for control of browning in rhizome explants were 0.56 mM for ascorbic acid, 0.52 mM for citric acid and 0.64 mM for L-Cysteine-HCl. A similar experiment carried out with leaf explants shown that 0.14 mM of ascorbic acid, 0.39 mM of citric acid and 0.16 mM of L-Cysteine-HCl was effective for control of browning.
Though all the three antioxidants have shown varying levels of control of browning, citric acid alone at 0.39 mM and 0.52 mM was more effective than other treatments imposed. These treatments had produced the lowest incidence of browning in addition to higher percentage of sprouting in rhizome (66.8 %) and leaf (66.6%) explants. Application of citric acid at 1.0 mM in MS media improved the shoot development from microshoots of *Prunus avinum* (Vasar, 2003). Furthermore, the antioxidant property of citric acid in reducing phenolic oxidation in tissue culture system has been well documented (Bhatt and Dhar, 2004). Use of antioxidants were reported to be effective in control of browning of explants in many species such as *Rhododendron* (Preil and Engelhardt, 1977), Walnut (Rodriguez, 1982), *C. zeylanicum* (Rai and Jagadishchandra, 1987), *Cattleya* sps (Ichihashi and Kako, 1977), and several other species. In support of these reports, use of antioxidants is very effective in control of browning in leaf explants of *C. orchioides*.

Based on the results obtained from the previous experiments, the following protocol is suggested for initiation of rhizome and leaf explants of *C. orchioides*

- Actively growing healthy rhizome and young leaves have to be collected.
- Rhizome and leaf have to be rinsed under running tap water for 10 min to remove the debris adhering on the surface.
- Soaking of rhizome and leaf in Tween 20 solution (5 drops in 500 ml water) for 10 min has to be followed.
- Rhizome and leaf have to be rinsed again under running tap water for 5 min.
- Rhizome and leaf segments have to be surface sterilized separately using 0.1 % HgCl₂ for about 15 min and 6 min respectively.
- Surface sterilized rhizome and leaves have to be rinsed using sterile distilled / deionised water at least 5 -7 times under aseptic condition.
Out growth such as adventitious roots and outermost tissues of rhizome have to be chopped.

Segments measuring 1 cm length in case of rhizome and 1 cm² in case of leaf have to be cut under sterile water and placed on sterile filter paper to remove the water before implanting on the sterile nutrient medium.

Shoot proliferation is a crucial stage which decides the application of micropropagation in any plant species. In this stage, large number of rootable shoots are produced for micropropagation and also to supply the cultures required for genetic stock. The success of shoot multiplication generally depends on various physiological factors of mother plants. It is well documented that the response of the explant can be altered by providing suitable culture conditions. The important factors that contribute for shoot multiplication are selection of suitable basal medium and use of suitable cytokinins at an appropriate concentration. In the present study, experiments were conducted with various growth regulators such as BAP, KN, 2, 4-D and NAA. In addition, different adjuvant such as silver nitrate and phloroglucinol were also tested individually or in combination with cytokinins to enhance shoot proliferation. Appropriate season for initiation and establishment of leaf cultures was identified.

Experiments carried out for optimizing the concentrations of BAP, KN, 2,4-D and NAA revealed that 0.44 µM, 4.4 µM of BAP and 0.46 µM, 4.6 µM of KN were effective for inducing high frequency multiple shoots. However, the number of bulbils per explant was ranging from 1.0 – 3.1 and the number of shoots per explant was varying from 1.0 – 5.6 depending upon the concentrations used. The microshoots regenerated in these combinations were significantly longer than the other combinations tested except for BAP and KN. Shoots regenerated in presence of BAP at 0.44 µM and 4.4 µM had attained the length of 11.5 cm but the higher concentration of BAP and KN had induced shorter shoots with 1.2 cm and 1.6 cm respectively. Thus, BAP at 0.44 µM was more suitable than other concentrations for inducing longer shoots in micropropagation of *C. orchioides*. However, the number of shoots obtained per leaf explant was very low even at higher concentration of various cytokinins either
alone or in combination with auxin. Faster response of leaf explants to BAP was very often observed in this experiment.

Qu et al., (2000) have reported that presence of higher concentration of cytokinins in the medium cause cytogenetic instability and often reported to be unsuitable for clonal propagation. In general, explants sourced from herbaceous plants are highly responsive to BAP and most of the cultures produced healthy shoots suitable for subsequent shoot proliferation and rooting in micropropagation (Debergh and Zimmerman, 1991). A number of reports are also available, where MS medium with different concentrations and combinations of BAP and NAA were used for high frequency shoot regeneration from different explants of Zingiber officinale (Bhagyalakshmi and Singh, 1988; Ikeda and Tanabe, 1989). It was reported that leaf explants produced somatic embryos or bulbils on media supplemented with 2, 4, 5-trichlorophenoxyacetic acid or IBA with cytokinins in C. orchioides (Suri et al., 1998). In liquid medium, regeneration of large number of bulbils was reported from leaf explants of C. orchioides (Jain et al., 2007). It was reported that both auxin and cytokinins are essentially required in combination for inducing shoot organogenesis in leaf culture of Cicer ariatum (Arockiaswamy et al., 2000). The present study also shown that, leaf explants of C. orchioides require a combination of auxin and cytokinins for regeneration of large numbers of bulbils and shoots. Thus, the previous findings reported in other plant species are in concurrence with the present findings.

Use of AgNO₃ in micropropagation of C. orchioides was found to be beneficial as this compound could enhance the overall response of leaf explants. Leaf explants cultured in presence of BAP alone (control) responded well for early response and shoot regeneration. However, highest percentage of response (65.5 %) was realized only when the medium was supplemented with BAP (4.4 µM) and AgNO₃ (0.12 mM). Of the various concentrations, AgNO₃ at 0.12 mM was found to induce maximum number of bulbils (6.8/explant) from leaf explants of C. orchioides. The beneficial role of AgNO₃ in micropropagation of several plant species have been realized and a number of reports on the use of this compound was available. It was reported that
AgNO₃ was effective at different concentrations ranging from 20 - 200mg/l (Bais et al., 2000; Ganesh and Sreenath, 2008; Noman et al., 2008; Kumar et al., 2009).

The beneficial effect of AgNO₃ in improving the regeneration and development of multiple shoots was reported by many workers (Purnhauser et al., 1987; Mallika et al., 1996; Ganesh and Sreenath, 2008). AgNO₃ was reported to be effective at various concentrations ranging from 0.12 - 12 µM (Ganesh and Sreenath, 2008; Noman et al., 2008; Kumar et al., 2009). Being AgNO₃ as potential ethylene inhibitor, addition of this compound plays a key role in inhibiting the synthesis of ethylene by the wounded explant as reported by Lentini et al., (1988). This compound improved the regeneration of many dicot and monocot cultures (Duncan et al., 1985). In the present investigation, AgNO₃ was highly beneficial with regard to regeneration of more number of bulbils, which is considered to be an important parameter since each bulbils give rise to either one or many microshoots.

Experiment carried out to investigate the influence of PG for improving the regeneration of bulbils and microshoots from leaf explants of Curculigo orchioides revealed positive result. Of the various concentrations of PG tested, PG at 0.63 mM and 0.79 mM induced maximum sprouting with 48 % and 60 % respectively. These two concentrations induced the highest number of bulbils per explant. Presence of PG (0.79 mM) along with BAP (4.4 µM) induced the highest number of shoots (6 shoots/explant) and adventitious roots (13 roots/shoot). In addition, microshoots produced on this medium had produced longer shoots (9.29 cm) than the other treatments. The beneficial role of PG in enhancement of plant regeneration was emphasized by many workers. Jones and co workers (Jones, 1976; James and Thurbon, 1979; Jones and Hopgood, 1979) have emphasized the importance of phloroglucinol in shoot multiplication and rooting in a number of rosaceous fruit cultivars. The promotive effect of PG was confirmed later by McComb (1978). Hunter (1979) successfully cultured different explants of Cinchona ledgeriana in presence of PG and its beneficial role in inducing morphogenesis was highlighted. Phloroglucinol is a phenolic compound, predominantly found in xylem sap of apple and is known to
promote growth and development in a number of plant species such as *Chinchona ledgeriana* (Hunter, 1979), rosaceous fruit trees (Singha 1980; Zimmerman and Broome, 1981) and Cocoa (Mallika *et al*., 1996). In the present investigation, PG was found to be beneficial in enhancing the regeneration of bulbils besides to produce high frequency multiple shoots.

In this study, combination of cytokinins (BAP and KN) and adjuvant (AgNO₃ and PG) was found to enhance the bulbils and shoot proliferation in leaf explants of *C. orchioides*. Presence of 4.4 µM BAP in combination with 0.12 mM AgNO₃ was found to be suitable for induction of maximum regeneration of bulbils in addition to faster shoot regeneration. But combination of 4.4 µM BAP, 0.12 mM AgNO₃ and 0.79 mM PG had induced the highest mean number of healthy bulbils per explant (13.4) but number of shoots produced in this combination was comparatively lesser than the previous treatment. The synergistic effect of AgNO₃ with BAP and KN on enhancement of shoot regeneration was reported in a number of species such as *Brassica campestris* (Chi and Pua, 1989) and *Nicotiana plumbaginifolia* (Purnhauser *et al*., 1987). The present study revealed that presence of 0.12 mM AgNO₃ in combination with cytokinins (BAP and KN) enhanced more number of bulbils as well as multiple shoot regeneration in leaf explants of *C. orchioides*. Therefore, it could be concluded that AgNO₃ acted synergistically with BAP and KN to enhance the shoot proliferation in leaf explants of *C. orchioides* as reported by previous workers.

Studies were also extended to determine the influence of auxin for enhancing the callus induction in leaf explants of *C. orchioides*. When, 2, 4-D was combined with AgNO₃ and PG, the highest percentage of callus induction could be achieved (50.8 %). Absence of PG and AgNO₃ did not induce any callus from the leaf explants but shoot regeneration without callus proliferation was noticed. This could be possibly due to presence BAP in the medium. It was very clear that PG and AgNO₃ enhance the callus induction as well as organogenesis. From this study, it could be concluded that all the adjuvant along with BAP acted synergistically to enhance the organogenesis and shoot proliferation. In support of these findings, it was reported that omission of any one of
the compounds did not give satisfactory results in a number of plant species such as *Theobroma cocoa* (Mallika *et al*., 1996), *Coffea arabica* (Ganesh and Sreenath, 2000), *Vitex negundo* (Mercy *et al*., 2010).

In the present study, the most suitable season for establishing microshoots using leaf explants of *C. orchioides* could be determined. Of the four seasons, South-West Monsoon (June - August) and North-East Monsoon (September - November) were found to be suitable for establishment of leaf culture due to less incidence of microbial contamination coupled with high frequency of multiple shoot proliferation. Mother plants of *C. orchioides* shown active vegetative growth during two seasons namely South-West monsoon and North-East monsoon. In the other two seasons, shoots were either at slow rate of vegetative growth or in complete dormant phase. Explants sourced during South-West Monsoon and North-East Monsoon had responded well and this observation could be correlated with active shoot development during the above two seasons. During the other two seasons, namely Intermediate (December - February) and Summer (March - April) poor response of explant was observed possibly due to slower vegetative growth of mother plant. The influences of seasons on bud break and shoot regeneration was reported in a number of plant species such as *Eucalyptus* (Das and Mitra, 1990) and *Coffea arabica* (Ganesh and Sreenath, 2002). They reported that effective bud break and shoot regeneration was seasonal dependent. More recently, culture of nodal explants of *Stevia rebaudiana* had shown maximum bud induction (80.5 %) only during June – August (Verma *et al*., 2011). Thus, the present findings are in concurrence with the findings of the previous reports.

Orientation of rhizome explants of *C. orchioides* in the nutrient medium was found to influence shoot regeneration in varying responses. It was found that the highest mean number of multiple shoots/explant (6.66) was obtained when rhizome segments were placed horizontally on the nutrient medium. Rhizome explants placed on the medium other than horizontal orientation shown lesser response with regard to the number of days required for response and number of shoots/explant (Table 20). A similar result was reported when stem explant of *Narcissus pseudonarcissus* was placed
horizontally on the nutrient medium with the highest frequency of response (Sage et al., 2000). In general, shoot tip, nodal and rhizome segments are placed on the medium by facing the distal end in contact with medium as reported in a number of plant species such as *Pisonia alba* (Jagadishchandra et al., 1999), *Dendrobium moschatum* (Kanjilal et al., 1999). In *C. orchioides*, rhizome explants were implanted by facing the distal end in contact with medium and adequate number of microshoots was produced (Augustine and Souza, 1997). But the present finding conclude that positioning of rhizome explant horizontally on the nutrient medium induce early response beside to produce more number of microshoots.

Experiment carried out with cytokinins (BAP and KN) in combination with auxin (2,4-D and NAA) for *in vitro* rooting of microshoots of *C. orchioides* had shown varying responses with regard to percentage of rooting and number of roots produced by microshoots. Presence of 4.4 µM BAP, 4.6 µM KN and 5.3 µM NAA had induced the highest percentage of rooting (76.6 %), with maximum number of roots/shoot (40.4). All the seven combinations of media had induced healthy and longer roots that were either brownish or white in color (Table 21). In *C. orchioides*, it was reported that emergence of roots during *in vitro* rooting occurred within 15 – 20 days and further maintenance of culture for another one week lead to vigorous root growth with the development of longer adventitious roots in presence of 0.53 µM of NAA (Wala and Jasrai, 2003). In *Curcuma longa*, microshoots produced from the rhizome were transferred from solid – liquid medium for enhancing the development of roots (Nadgauda et al., 1978). But, in the present study, large numbers of microshoots were converted into complete plantlets with healthy root system without transfer of microshoots from solid to liquid medium, avoiding an addition step to reduce the cost of production.

Plantlets regenerated from rhizome and leaf explants of *C. orchioides* were separately transferred into various substrates containing soil, sand and vermicompost in different ratios (Table 22 and 23). Of the various planting substrates, two combinations of substrate consisting of soil, sand and vermi compost (3:3:3 and
shown the highest survival percentage of plantlets derived from rhizome (96.4 %). Whereas, plantlets regenerated from leaf tissues had shown 81 % survival in the substrate containing soil, sand and vermicompost (2:2:5). It was reported that high humidity was essential for maintenance of plantlets by spray of water at a regular intervals during *ex vitro* acclimatization of plantlets of Banana (Jasrai et al., 1999) and *C. orchioides* (Jasrai and Wala, 2000). It was also reported that plantlets of *C. orchioides* survived at a high frequency (96.4 %) during hardening and showed a vigorous growth of rhizome and roots without any morphological variations (Wala and Jasrai, 2003). In the present study, *ex vitro* establishment of plantlets regenerated from leaf and rhizome explants of *C. orchioides* was found to be easier as revealed by the highest percentage of survival with minimal care.

5.2. Molecular characterization of micropropagated plants of *C. orchioides*

In order to determine the genetic fidelity of plantlets regenerated from five different pathways of micropropagation in *C. orchioides*, 10 ISSR primers were designed based on the primer sequences of closely related species. Of the 10 ISSR primers, four primers (Co-ISSR-03, Co-ISSR-05, Co-ISSR-07 and Co-ISSR-09) produced the maximum number of scorable bands (10), monomorphic bands (6) and polymorphic bands (4). It was reported that ISSR regions lying within the range of microsatellite repeats have a higher capacity to reveal polymorphism and offer great potential to determine intragenomic and intergenomic diversity as compared to other arbitrary primers such as RAPD (Zietkiewicz et al., 1994). The ISSR primers are now proven to be much more efficient in assessing the genetic integrity among clonally propagated plants of different species (Zietkiewicz et al., 1994; Bhatia et al., 2009; Mohanty et al., 2010; Bhatia et al., 2011). Martins et al., (2004) reported genetic homogeneity of almond plantlets regenerated through axillary branching after 4 - 6 years of *in vitro* multiplication.

Homogeneity was reported in micropropagated plants of *Swertia chirayita* employing ISSR marker (Joshi and Dhawan, 2007). ISSR markers revealed higher levels of polymorphism than RFLP markers in maize (Kantety et al., 1995) and also
useful for detecting somaclonal variation among micropropagated plants of coffee (Rani et al., 2000). It was reported that ISSR analysis revealed monomorphism among the micropropagated plants of Rauvolfia serpentine and found to be genetically stable (Saravanan et al., 2011). But the present finding concludes that micropropagated plants from five different pathways of C. orchioides produced the maximum number of monomorphic bands, revealing their genetic integrity.

5.3. Phytochemical investigation of micropropagated plants of C. orchioides

Thin Layer Chromatography of rhizome extracts obtained from normal and micropropagated plants of five different pathways revealed five similar prominent TLC spots in case of plantlets obtained from pathways 1, 3, 4 and 5 with respective Rf values (0.18, 0.20, 0.36, 0.39 and 0.71). These spots were comparable with control as they found to have similar Rf values. However, rhizomes from plantlets regenerated through pathway 2 had shown only four prominent spots as similar to the above but with the absence of one band having the Rf values of 0.71 in other samples. A similar technique was employed for determining the phytochemical properties of root extract of micropropagated plants of Withania somnifera and confirmed that the micropropagated plants were similar to that of normal one (D’Silva and Senarath, 2009). The present study proven that TLC is a simple and cost effective method to evaluate the presence of major phytochemical in C. orchioides.

Comparative study of rhizomes obtained from normal and micropropagated plants of C. orchioides was carried out by performing HPLC finger prints. This experiment revealed four common peaks from the rhizome of normal and micropropagated plants. It was reported that if the relative standard deviation (RSD) of retention time of common peaks for different samples is less than 1 %, these peaks are generally referred to be the same compound. This analysis was already reported in Erigeron breviscapus (Lie et al., 2004; Liu et al., 2007). It was reported that if the RSD of relative peak area of common peaks is more than 10 %, it can be referred as different compounds as previously reported in E. breviscapus (Liu et al., 2008). However, Akarasereenont et al., (2010) have reported that if the RSD of relative retention times
of common peaks from 1 % to 10 %, the compounds can be considered as same phytochemically. Thus the four peaks noticed in the HPLC chromatogram of rhizome obtained from micropropagated and normal plants were considered as same phytochemically since the RSD values were less than 1 %.

In the present study, HPTLC finger prints developed from the rhizome of plantlets obtained through five different pathways of micropropagation shown similarities with respect to their major phytochemical constituents (Table 25). HPTLC chromatographic finger print displayed the various types of flavanoids such as rutin, ferulic acid, caffeic acid and quercitrin from the rhizome of all the samples. However, the quantity of these compounds was higher in the rhizomes of plantlets regenerated from pathways 1 and 5 and comparable with control. Among the four flavanoids, the quantity of rutin was higher in the rhizome of plantlets regenerated from pathways 1 and 5 (5.44 µg/g and 5.55 µg/g). It was reported that the percentage of rutin was higher than that of quercitrin in the leaves of *Tephrosia purpurea* (Jain et al., 2009). It was reviewed that the HPTLC technique can be used to solve many qualitative and quantitative analytical problems in a wide range of fields (Weins and Hauck, 1996).

The present study concluded that rhizomes of plantlets regenerated from five different pathways of micropropagation of *C. orchioides* contained similar flavanoids as mother plant and suggested that these protocols can be used for large scale propagation to meet the demand of *C. orchioides* in pharmaceutical industries.
Fig. 23: General protocol developed for regeneration, multiplication and acclimatization of plantlets derived from leaf and rhizome explants of *C. orchioides*. The duration required for shoot multiplication and *ex-vitro* establishment of plantlets was 90 days and 60 days respectively.