1. In vitro callus induction of *Alternanthera sessilis* and *Scoparia dulcis*

In general, same surface sterilization protocol is being followed for different explants of same herbs (Mungole *et al.*, 2009; Biswas *et al.*, 2010; Mungole *et al.*, 2011). In the present study, different surface sterilization protocols were studied among which 0.1 % HgCl₂ was the most effective surface sterilization for both leaf (Table 4) and nodal explants (Table 6). In tissue culture, depending on other hormones present in the medium, changes in auxin concentrations may change the type of growth, e.g., stimulation of root formation may switch to callus induction etc. IAA is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation and responses to light and gravity (Lebuhn and Hartmann, 1993). Although the callus induction and plant regeneration of *Scoparia dulcis* and *Alternanthera sessilis* have been documented, there is still much to be learned about the effects of IAA on morphology and physiological parameters. Addition of IAA for callus induction increased both survival rate and callus mass in both *Alternanthera sessilis* and *Scoparia dulcis* (Table 5). This supports the fact that mixture of more than one auxin can be effective for callus induction and a mixture of a synthetic auxin and IAA has been already found by many workers to be more effective than the synthetic compound on its own (Nabors *et al.*, 1983). Nabors *et al.*, (1983) also found that a mixture of 2,4-D (or 2,4,5- T) and IAA was found to promote friable callus formation in wheat, pearl millet and some varieties of rice. Similarly in the present study also combination of 2,4-D and IAA induce more friable callus than 2,4-D alone. Dicotyledonous callus, or suspension cultures requiring auxin (e.g. 1 mg/l IAA) but not cytokinin for growth (Syono and Furuya, 1972). In the present investigation also, there is no cytokinin is used for callus induction.
2. In vitro plant regeneration of Alternanthera sessilis and Scoparia dulcis

The auxin:cytokinin ratio represents an important signal in the formation of cell phenotype and also in the onset and maintenance of the process of cell division (Stickens et al., 1996). The ability of auxins (together with cytokinins) to manage key events in plant morphogenesis was documented, among others, by Skoog and Miller’s (1957) discovery of the regulation of organogenesis in vitro by means of the auxin:cytokinin ratio in culture media. In the present investigation, combination of 0.5 mg/l KIN and 2.0 mg/l IAA gave the maximum response in respect of number of shoots and roots simultaneously from nodal explants of Scoparia dulcis (Plate 5; Table 7). This correlation in results can be attributed to many factors, however Murashige and Skoog (1962) stated that the presence of casein hydrolysate allowed vigorous organ development over a broader range of IAA and KIN levels. Zapata et al. (1983) reported that pith phloem callus of tobacco forms shoots on MS medium containing 10^{-5} M IAA and 10^{-5} M KIN. Boxus and Terzi (1988) advocated that the addition of 0.5 mg/l KIN and auxin to the rooting medium for strawberries and several woody plants, found that at this concentration, the cytokinin had a bacteriostatic effect and rooting was not impaired.

In case of Alternanthera sessilis, combination of 0.5 mg/l BAP and 0.2 mg/l IAA gave the maximum response in respect of number of shoots and roots (Plate 6; Table 7). Instead of IAA, IBA has also been used in many experiments. BAP is an effective cytokinin for shoot initiation and elongation. BAP promoted axillary bud proliferation of Castanea in the experiments of Vieitez and Vieitez (1980), whereas KIN was not effective against that host. Elliott (1970) found KIN was incapable of promoting the growth of rose shoot tips. On the other hand, only 0.5-5 mg/l KIN (together with gibberellic acid) induced the proliferation of potato shoots, where BAP and 2-iP were not effective. BAP gives a high rate of shoot proliferation in Gerbera, but the best shoot quality is obtained using 5-10 mg/l KIN (Pierik et al., 1982; Hempel et al., 1985). Auxin and cytokinin became essential additions to Linsmaier and Skoog (1965) medium to produce bulblet, callus and root formation from explants cut from the distal (and normally non-regenerative) part of Lilium longiflorum bulb scales (Dennis and Ascher, 1976).
In the present experiment, the range of 0.2 – 2 mg/l IAA was used under light irrigation (16 hours photoperiod) for in vitro plant regeneration. Marcotrigiano and Stimart (1981) found that in the light, hypocotyls of Paulownia required 3 mg/l IAA in the medium to produce shoots at the maximum rate, whereas under continual darkness only 1 mg/l IAA was necessary. Cytokinin (KIN, 3 mg/l) was required at the same concentration under both regimes.

In conclusion, these results show that nodal explants have the potential for direct regeneration. The protocol developed is simple, reproducible and yields true to true type plants. Crop improvement by traditional methods (mass selection, inbreeding and hybridization) is labour intensive, time consuming, strongly influenced by environmental conditions and depends on the existing gene pool(s). In contrast, callus culture offers tools for genetic cell transformation, through somaclonal variation, induced mutagenesis and genetic engineering which are not only much more rapid than conventional breeding but can also give rise to novel genes and genotypes. The direct regeneration system developed in this study would provide a step towards the application of such methodology, for regeneration of other important medicinal plants under in vitro conditions.
3. Diversity of fungal contaminants from plant tissue culture of *Alternanthera sessilis* and *Scoparia dulcis*

Despite the best timing and selection efforts it is almost impossible to eliminate contamination from *in vitro* grown plants. In fact according to (Leifert *et al.*, 1991) losses due to contamination *in vitro* average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories, the majority of which is caused by fungal, yeast and bacterial contaminants (Leifert *et al.*, 1994). The present investigation concentrates on fungal contaminants and was recorded between 4.5 to 5% from the overall experimentation.

During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji *et al.*, 2009). Based on this fact the surface sterilization was standardized to 0.1 % HgCl₂ with the highest survival rate (94 % in *Scoparia dulcis* and 93.2 % in *Alternanthera sessilis*).

In previous reports, most of the overt contamination from nodal explants occurred during the first two weeks after initiation (Mitchell *et al.*, 2003). However in the present experiment the cultures were monitored for two weeks from day of inoculation for the occurrence of fungal contaminants. Use of antimicrobial chemicals (such as antibiotics) may cause phytotoxicity, retard explants growth and encourage the build up of resistance. Furthermore, even though some antibiotics may give comparatively high activity when tested on defined bacteriological media these results are not usually replicable on the complex tissue culture media and so the expected results are usually elusive (Barrett and Cassells, 1994).

More contaminants were observed in leaf than nodal explants (Table 8). Similarly, more endophytic fungi were reported in leaf than petiole segments in *Azadirachta indica* (Rajagopal and Suryanarayanan, 2000) and palm tree (Rajagopal, 2004). Such variation within leaf of endophytes is attributed to differential leaf expansion and leaf chemistry (Bills, 1996). Exceptionally *Penicillium verruculosum* has comparatively higher colonization frequency in
nodal explants than leaf explants. *Penicillium verruculosum* has already been reported as a root endophytic fungus of *Potentilla palgens* (Bhagobaty and Joshi, 2009).

In the present study, all the three fungal contaminants of *Scoparia dulcis* such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, and *Penicillium citrinum* were isolated as endophytes (Table 11). Many endophytic fungi have been identified from plant tissue cultures including species of *Alternaria*, *Acremonium*, *Aspergillus*, *Candida*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Helminthosporium*, *Microsporum*, *Penicillium* and *Tricothecium* (Subathra Devi and Mohana Srinivasan, 2006). Hence, endophytic fungal contaminants are ineradicable from plant tissue cultures.

Fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions, and are now routinely used in phylogenetic studies, as well as in the detection and identification of fungi (Guo *et al*., 2000, 2001; Arnold *et al*., 2001; Okane *et al*., 2001; Baayen *et al*., 2002; Lord *et al*., 2002; Anderson *et al*., 2003; Jeewon *et al*., 2004).

Since the currently available DNA extraction protocols are rather costly and time consuming (Sambrook and Russel, 2001), we adapted a rapid DNA isolation method from plants (Gonzalez-Mendoza *et al*., 2008) combining chemical reagent digestion without mechanical shearing for lysing the hyphae of fungal strains followed by DNA isolation. The omission of maceration is the major advantage of this DNA isolation procedure that reduces sample handling and minimizing the risk of contamination between samples. This is particularly important in work involving amplification by PCR. Additionally, this protocol provides a rapid, reliable, and low-cost alternative to the existing DNA purification protocols used in research and clinical laboratories (Liu *et al*., 2000; Pfaza *et al*., 2004; Gonzalez-Mendoza *et al*., 2010). Therefore, the protocol is an efficient (<2 h) and inexpensive procedure for the isolation of good-quality DNA from fungal strains for molecular assays. In all samples, the A260/230 values were higher than 1.8, suggesting negligible contamination by polysaccharides (Gonzalez-Mendoza *et al*., 2010).

Endogenous bacterial contaminants isolated from infected cultures of *Ilex dumosa* nodal segments were identified as *Stenotrophomonas maltophilia* and *Achromobacter* sp. using 16S
rDNA analysis (Luna et al., 2008). In this study, 18S rRNA gene was used for the detection of fungal contaminants in plant tissue culture using characterized reference strains. The primers are based on the conserved region of 18S rRNA gene which is designed to detect wide range of fungal strains (Embong et al., 2008). The findings of this study agree with those of Lohmann et al., (1998) and Anand et al., (2001), demonstrating high analytical specificity and sensitivity of the PCR method compared with the conventional method.

Generally endophytic fungal isolates can only be identified based on morphological characteristics if they sporulate on the media. Despite the development of various methods to promote sporulation, e.g. by growing them on modifications of artificial media and under various incubation conditions (Guo et al., 1998; Taylor et al., 1999), the number of isolates that do not sporulate ranges from 4.5 to 54% (Petrini et al., 1982, Espinosa-Garcia and Langenheim, 1990; Johnson and Whitneey, 1992; Fisher et al., 1993; Guo et al., 2000; Photita et al., 2001; Cannon and Simmons, 2002; Kumaresan and Suryanarayanan, 2002). Since conventional classification of fungi relies heavily on reproductive structures, these non-sporulating mycelia sterilia cannot be provided with taxonomic names. In order to appreciate the considerable diversity of these mycelia sterilia in culture, they are generally categorised as ‘morphotypes’ based on similar cultural characters (Taylor et al., 1999; Frohlich et al., 2000; Guo et al., 2000; Arnold et al., 2001; Cannon and Simmons, 2002). This approach has been shown to be justifiable and related to the diversity of fungal contaminants.

Guo et al., (1998) reported 52.2 % of non-sporulating isolates even after incubation methods to promote sporulation. In a survey of the endophytic fungi from Pinus tabulaeformis in northeast China, approximately 11% of isolates did not produce spores, although various techniques were employed to promote sporulation (Wang et al., 2005). Similarly in the present study even after several attempts to produce spores, the sterile form I did not sporulate. If the isolates do not produce any spores in culture they cannot be identified using morphological taxonomy, and therefore molecular techniques have been employed to identify such fungi (Arnold et al., 2000; Guo et al., 2000, 2001; Okane et al., 2001; Baayen et al., 2002).
A major limitation demonstrated in previous studies was the limited number of sequences in GenBank and EMBL (Guo et al., 2000, 2001). However, as more sequences become available this identity of more taxa can be revealed. The sterile mycelia morphotypes in this study that were given for BLAST algorithm to find the highest similarity sequences. Fortunately there are more similar sequences which have been found in NCBI-GenBank that correspond to sterile form I and hence it was identified successfully. The 18S rRNA gene sequence of sterile form I (strain PTC01) displayed 99% identity with Colletotrichum gloeosporioides isolate XSD-139 (EU326190.1) and Colletotrichum gloeosporioides strain HBwh-2. Hence, sterile form I (strain PTC01) should be identified as Colletotrichum gloeosporioides.

In the present study, other fungi, i.e. Aspergillus niger, Aspergillus tamarii and Penicillium citrinum have been identified by morphological methods, however molecular techniques were used to confirm the same. Alternaria alternata, Fusarium lateritium, Penicillium verruculosum and Colletotrichum gloeosporioides were identified at genus level by using morphological methods and characterized at species level by using molecular techniques.

The results indicate that molecular techniques are promising methods to identify fungal strains. The identity of fungal strains was confirmed by both morphological and molecular methods. This study has also demonstrated the occurrence of endophytic fungi as fungal contaminants in plant tissue cultures.
4. Screening, extraction and quantification of IAA from fungal contaminants

Many fungi can produce auxins in axenic cultures (Buckley and Pugh, 1971; Gruen, 1959). Bacterial strains, such as *Pseudomonas, Erwinia*, and *Agrobacterium* were already reported for the production of IAA (Comai and Kosuge, 1982; Costacurta and Vanderleyden, 1995; Manulis *et al.*, 1991). From the results of the present study, it was clear that all fungal contaminants screened for IAA production (Table 12; Graph 1), are capable of synthesizing IAA in culture medium with the addition of tryptophan as a precursor.

IAA appears to have an enhancing effect on processes such as sporulation, spore germination, and germ-tube elongation (Robinson *et al.*, 1998). This may be the reason for lesser production of IAA in sterile form I, *Colletotrichum gloeosporioides* PTC01 (51.1 µg ml\(^{-1}\)) than sporulating strain *Colletotrichum gloeosporioides* PTC03 (53.2 µg ml\(^{-1}\)) (Table 12; Graph 1). Although fungi are capable of producing IAA, however, we present evidence that IAA promotes sporulation and causes changes in growth morphology in the fungal contaminant, *Colletotrichum gloeosporioides*. These results suggest the importance of IAA to fungal development and reproduction. Already fungal production of IAA is reported for its interactions with host plant (Shinshi *et al.*, 1987; Yamada *et al.*, 1985).

GC-MS provides precise measurements of IAA, with the accuracy of each estimate being checked by comparing response ratios of several characteristic fragments (Cohen *et al.*, 1986; Petersson *et al.*, 2009). The GC-MS spectrum of fungal sample (Plate 32) with respect to different retention times gives peaks with considerable relative abundance according to characteristic fragmentations. The spectrum of fungal IAA similarly matches with standard (Plate 31) with respect to different retention times which corrolatates with the results of Edlund *et al.*, 1995.

Overall results reveal that *Colletotrichum gloeosporioides* was the highest producer of IAA. Some of the symptoms caused by *Colletotrichum gloeosporioides* have been already recorded, e.g., epinasty and leaf deformation, are mimicked by exposing plants to IAA (Robinson *et al.*, 1998). The identification of the enzymatic reactions involved in IAA
biosynthesis provides a basis for the cloning of IAA biosynthesis genes from *Colletotrichum gloeosporioides* and determination of the role IAA may play in fungus-plant interaction (Robinson *et al.*, 1998). Further, induction by IAA does not necessarily imply that it would be involved in IAA transport, especially because *Colletotrichum gloeosporioides* produces large quantities of IAA, so induction might be through endogenous rather than exogenous IAA. In addition, IAA-responsive gene CgOpt1 which appears to be involved in mediating IAA's effects (Veronique *et al.*, 2009). At this stage however, the underlying mechanism is unknown and further investigation is needed. Also the IAA production is essential to be utilized for commercial plant development.

5. Application of Indole-3-acetic acid in plant tissue culture: Comparative analysis

The culture broth containing 10 µg/ml IAA from *Pencillium verruculosum* RS7PF (EU579531), a fungal endophyte showed the ability to promote seed germination in *Vigna radiata* and *Cicer arietinum* (Bhagobaty and Joshi, 2009). Except this study of using fungal IAA for plant germination, there appears to be no others report available on utilization of fungal IAA for plant tissue culture experiments and especially for callus formation. *Pseudomonas putida*, an endophytic bacteria produced 22.5 µg/ml of IAA and it was utilized for the development of the host plant root system (Patten and Glick, 2002). Although minor morphological variations were observed with *Alternanthera sessilis*, morphologically similar results in plant regeneration were observed with the fungal IAA as like control IAA.

The determination of the carbohydrate level in callus reveals the readily available source to the plant growth and has several applications in physiological studies (Passos, 1996). Sugar levels analysis has application in several physiological studies because it reveals reserve levels promptly available to the growth (Passos, 1996). Growth curve was established for callus of *Tabebuia roseo alba* by determining the contents of TSS, RS, total soluble proteins and amino acids (Abbade *et al.*, 2010). In order to compare efficacy of fungal IAA at physiological level, TSS, RS, amino acid and proteins were analyzed in callus tissues induced with authentic and fungal IAA (Table 17; Graph 2). There is not much variations were recorded in physiological parameters such as TSS, RS, amino acid and protein. Although amino acid content is slightly
decreased in callus cultures of *Scoparia dulcis*, other parameters such as TSS and RS did not show notable variations. Slightly increased TSS content was observed in callus culture of *Alternanthera sessilis*. Only negligible amount of protein was recorded from all the callus tissues irrespective of IAA source (Table 17). This indicates that probably the synthesis of the amino acids occurred from the protein degradation (Serra *et al.*, 2000). This increase can be attributed to proteolysis, because the decline of leaf protein concentration. Amino acids when supplied at sufficient concentrations can be used for synthesis of protein (Dougall, 1966). Furthermore, the increase in the amino acid content can be occasioned by the considerable absorption of the ammonium ion and glycine from the culture medium. According to George *et al.*, (1988), the presence of ammonia in the culture medium leads to an increase of amino acid and protein synthesis, which are produced using the energy liberated from the carbohydrate catabolism. This decrease also suggests that the callus tissue possibly would have used the protein reserve of the explants during earlier storage of callus dedifferentiation (Abbade *et al.*, 2010).