2. Review of literature

2.1. The orchid seeds

Orchid seeds are among the most striking characteristics of the *Orchidaceae* family. They are minute, exceedingly small and powdery, weigh from 0.3 to 14 µg. (Burgeff, 1936; Harley, 1951) and measure from 0.250 to 1.2 mm. in length (Knudson, 1922) and 0.090 to 0.270 mm. in width (White, 1927; Knudson, 1929). They are produced in large numbers ranging from 1,300 to 4,000,000 seeds per capsule (Harley, 1951; Arditti, 1961).

The great majority of orchid species have relatively undifferentiated seeds, non-endospermic or lack of cotyledons (Harley, 1951; Maheshwari and Narayanaswami, 1952). The embryo enclosed within more or less transparent coat by means of several fine strands or cells (Arditti, 1992). The undifferentiated embryo consists of dense granulated cytoplasm and conspicuous nuclei. Upon close examination, two distinct regions may be discerned. The posterior region consists of decidedly smaller and denser cells. It eventually develops into the apical meristem of the young seedling (Carlson, 1935; Burgeff, 1936). The suspensor, consisting of very large elongated and probably dead cells (Burgeff, 1936), can be seen attached to the posterior end of the embryo.

2.2. Asymbiotic germination

Orchids seeds, due to lack of metabolic machinery and functional endosperm, natural germination is very poor. Only 0.2-0.3% germinates in natural condition (Prasad and Mitra, 1975). For germination in natural condition, they require a
mycorrhizal association, which may be provided under natural undisturbed conditions (Cherevhenke and Bohalyr, 1984).

The ability of orchid seeds to germinate asymbiotically \textit{(in vitro)}, demonstrated for the first time by Knudson, (1922), in \textit{Cattleya}. Since then a large number of species and hybrids of diverse habits and habitats have germinated \textit{in vitro} without the intricacies of host-fungus relationships (Arora, 1990; Devi \textit{et.al}., 1997). Orchids seeds have been successfully germinated asymbiotically by several investigators from time to time (Arditti \textit{et.al}., 1982a; Gangaprasad \textit{et. al}. 1999; Vij, 1995; Vij and Pathak, 1988; Vij \textit{et.al}., 1981). The success of embryo culture indicated that orchid embryos become viable and are capable of normal development before they become fully ripe.

2.3. Seed germination of \textit{Vanda}.

Hegde and Sinha, (2002), reported that \textit{Vandaceous} orchids being essentially monopodial habit has been found to be very difficult to propagate vegetatively. Rao \textit{et. al}., (1998), used IY basal media for germination of seeds of \textit{V.coerulae}. He also worked on seed germination of \textit{V. coerulescens} and reported that Vacin and Went medium was most suitable to obtain seedlings directly from the seed protocorms. Roy and Banerjee, (2002), used three different media (viz. Ku C, VW and half strength MS), enriched with combinations of organic supplements for germination of seeds of \textit{V. tessellate}. Out of three, the best response of seed germination was found on Ku C supplemented with peptone 0.2%. Mathews and Rao, (1980), cultured pods of three interspecific \textit{Vanda} hybrids in four different types of basal media supplemented with different growth hormones. They recorded 100% germination in
two different basal media out of four. Holttum, (1964), reported that for germination of *Vanda* seeds, acidic medium was essential.

**2.4. Seed germination of *Dendrobium***.

Raghuwanshi *et. al.* (1986), studied *in vitro* seed germination of *Dendrobium nobile*, *D. chrysanthum* and *Sarcanthus pallidus* in modified Kn C basal medium. They studied the effect of different pH levels of nutrient medium on seed germination and maximum germination was achieved at pH 4.0 in case of *D. nobile*. Hazarika and Sarma, (1995), reported that 90% seeds of *D. transparens* germinated *in vitro* in MS medium supplemented with Kn and NAA (1.0 mg/l each). Kaur, (1996), worked on *D. densiflorum* and reported that for germination of seed, this species did not require cytokinin, as the species had got high endogenous level of cytokinin.

The role of auxins and cytokinins on germination of seeds had been variously assessed by different authors in various orchids. Incorporation of BAP in the media impaired the germination response and it suppressed the formation of absorbing hairs in *D. chrysotoxum* (Arora, 1990). He also reported that incorporation of Kn in the medium delayed the seed germination and subsequent seedling growth of *D. chrysanthum* and *D. denudans*. Its inhibitory effect on seed germination of *D. hybrid* was also reported by Kano, (1965). He reported that at higher concentration of GA$_3$, inhibited the germination of seeds of *Dendrobium*. Addition of 20% coconut water in the nutrient media favoured the germination of seeds and growth of embryo in *D. aqueum*, and *D. heterocarpum* (Arditti and Ernst, 1984). The beneficial effect of coconut water on advancement of germination as well as protocorm multiplication
was reported by Kher, (1999), in *D. chrysotoxum*. Devi *et al.*, (1990), used coconut water in the media and found that it favoured the seed germination as well as seedling growth of *D. species*.

### 2.5. Seed germination of *Paphiopedilum*

Prakash *et al.*, (1997), inoculated seeds of *Paphiopedilum fairieanum* on Burgeff N3f medium fortified with only 1% sucrose and resulted 50% germination of seeds while germination was not observed on KC medium and only 10% germination was recorded on VW medium. Early swelling and development of PLB’s were obtained from matured green pods of *Paphiopedilum lawerenceanum* XP. Winston Churchill cultured by Nagaraju and Upadhyaya, (2002a), in Nitsch medium supplemented with 1.0 mg/l BAP. In case of *Paphiopedilum insigne*, none of the media tested was found suitable. Arditti and Ernst, (1984), reported that incorporation of AC in the media enhanced the seed germination and growth of seedlings of *Paphiopedilum*. The medium fortified with coconut water promoted early germination as well as protocorm multiplication in *P. spicerianum* as reported by Kanika, (1998). Yam and Weatherhead, (1988), used the coconut water in the media and found that it favoured the seed germination of *P. purpuratum*. Thomale, (1954), reported that a combination of glucose and fructose was found necessary for the germination of *Paphiopedilum* seeds. He also reported that incorporation of peptone in the nutrient media accelerated growth of seedlings of *Paphiopedilum*. Kano, (1965), reported the inhibitory effect of light on germination of seeds of *P. callosum* and *P. spicerianum*. Sharma, (1996), tested the suitability of various sugars (glucose, fructose, maltose, sucrose etc.) at different concentrations in supporting
optimal germination in *P. spicerianum* and observed that sucrose at 2-3% gave optimal results.

**2.6. Effect of age on seed capsule**

The germination potential of embryos, at different intervals after pollination, in a large number of species suggests that the earliest stage at which the embryos can germinate is species specific. So, detection of the earliest stage of embryo development at which the embryos can be germinated in artificial media is important. However, very young orchid embryos fail to germinate and hence do not form suitable explants, due to dormancy, pH and other metabolic factors, as suggested earlier by Withner, (1953). The immature embryos, in general, germinate readily, and much better than the mature ones. The germination potential of the immature seeds was found to be directly correlated with their physiological age. The mature seeds, on the other hand, either fail to germinate or germinate very poorly probably due to loss of growth promoting factors and accumulation of inhibitory or dormancy factor, besides a change in the quality of food reserves (Burgeff, 1954; Kano, 1965; Stoutamire, 1974; Withner 1959). Other reasons for poor germination of mature seeds includes increase in the hydrophobic nature of the seed coat, lack of viability, presence of germination inhibitors in the mature seed coat and onset of dormancy in the mature seed (De Pauw and Rempherey, 1993). In *Dactylorhiza maculate*, the abscisic acid contents are much higher in mature seeds as compared to immature ones, may be responsible for seed dormancy (Van der Kinderen, 1987). Burgeff, (1954), reported that production of inhibitors during maturation of seeds of *Paphiopedilum*, which inhibited the germination of normally mature seeds.
2.7. **Nutrient media**

The germination response of seeds and their subsequent development into seedlings depends greatly on nutritional requirement. A large variety of culture media (Arditti *et al.*, 1982b; Burgeff, 1936; Curtis and Nichol, 1948; Murashige and Skoog, 1962; Knudson, 1946, Raghavan and Torrey, 1963; Thomale, 1954; Vacin and Went, 1949), differing in quality and quantity of their major and minor salts and in the presence/absence of vitamins have been devised on more or less empirical basis and many of these are species specific (Arditti *et al.*, 1982b). Liddell, (1944), observed that seeds of *Colopogon tuberosus* germinated rapidly in liquid Knudson B medium. According to Ichihashi, (1987), the quality and quantity of inorganic ions in the medium markedly affects the germination frequency *in vitro*. The choice of medium depends much on the type of orchid whose seeds are to be shown for germination. Sharma and Tandon, (1987), reported that Knudson’s C medium was found better for germination of some epiphytic orchids as compared to Pfeffer’s and VW media. According to Withner, (1959), a universal orchid medium is yet to be formulated since much is not known about the minimum mineral requirements of all the species.

2.8. **Effect of auxin and cytokinin on growth of seedling**

The benign effect of auxins and cytokinins on seed germination and protocorm development, when supplied exogenously had been reported by various authors in several orchids (Arditti and Ernst, 1984). The effect of Kn, when used singly in the medium, vary with the species and the medium formulation. It was effectively used to induce protocorm multiplication in *Cymbidium eburneum*
(Mahant, 1991). Hadley, (1970), reported that incorporation of Kn in the medium retarded seed germination of *Coeloglossum viride* and *Olatanthera longifolia*. On the other hand, beneficial effect of Kn in the medium, on germination of seeds of *Aerides multiflora, Pholidota articulate* and *Satyrium nepalense* were reported by Pathak, (1989). Borriss, (1969), reported that incorporation of BAP in the medium enhanced the germination frequency in *Cymbidium calceolus* and stimulated protocorm multiplication and shoot formation in *Cattleya aurantiaca*. Besides BAP and Kn, incorporation of GA$_3$ in the medium to enhance germination of seeds was also reported by many authors in different orchids from time to time. Perusal of literature revealed that GA$_3$ induced rapid germination in *Cattleya, Cyperidium, Cymbidium,* and *Odontoglossum* but protocorm in these taxa remained pale green in colour and failed to differentiate further (Humphreys, 1958).

Reports on incorporation of various auxins in the medium towards germination as well as proliferation of embryo were also available. By incorporation of IAA, at a very low concentration (0.1 mg/l), Prasad and Mitra, (1975), recorded 80% seed germination in *Cymbidium mastersii*. Vij *et al.*, (1981), reported that IAA at (0.25 mg/l) was found effective in case of *Vadaceous taxa*. Sharma and Tandon, (1986), also reported that at lower concentration of IAA (0.1mg/l) in the media, promoted seed germination and seedling growth of *Coelogyne punctulata*. On the other hand, its inhibitory effect on germination had also been reported by Hadley, (1970), in *Orchis purpurella*. Like IAA, presence of NAA in the media also stimulated the germination and seedling growth in several species like *Cattleya*
warheri (Mayer and Pelloux, 1948), *Epidendrum nocturum* (Yates and Curtis, 1949). Its inhibitory effects had also been reported by Goh (1970), in *Vanda Miss Joaquim*.

2.9. Effect of complex additives

In addition to standardizing media for germination of seeds of different species of orchids, effect of different complex additives (viz. coconut water, banana pulp, peptone, yeast extract etc.) were equally important. For germination and growth of protocorm, the used of coconut water had been reported by various authors widely in many orchid species (Hegarty, 1955; Nimoto and Sagawa, 1961). It not only enhanced the germination frequency but also promoted seedling growth. Like CW, incorporation of banana pulp (BP) in combination with other additives in media had also been recommended by Arditti (1977). Anderson, (1967), found that addition of 2-6 ml of ground banana pulp in Knudson’s C medium stimulated the growth of *Cattleya* seedlings. Chennaveeraiah and Patil, (1975), reported that incorporation of casinhydrolysate, CW, 2-4-D, IAA and NAA were essential for maximum germination of seeds of *Spathoglottis plicata*. Like CW and BP, addition of yeast extract has also been successfully used for seed germination and protocorm proliferation in many orchid species (Curtis, 1947b; Flamee, 1978; Mariat, 1948; Mathews and Rao, 1980; Prasad and Mitra, 1975). Vij et al., (1995), reported that yeast extract 1.0(gm/l) and kinetin 1.0(mg/) enhanced germination of unripe capsules of *Dactylorhiza hatagirea*. Likewise, incorporation of peptone along with banana extract promoted effective growth of protocorms of *Cymbidium* as reported by Kusumoto and Furukawa, (1977). Kulkarni and Surwase, (1998), reported that seeds of *Aerides maculosum* germinated readily on peptone enriched MS medium. To
improve the germination potential as well as to check the release of phenolic exudates, incorporation of activated charcoal (AC) in the media has been reported by many investigators. It had been reported to be beneficial for promoting germination frequency in large numbers of orchid species including *Bletilla striata*, *Eulophia sinensis*, *Spathoglottis pubescens* (Yam and Weatherhead, 1988). Vij and Pathak, (1988), reported that supplementation of 0.2% activated charcoal proved beneficial in checking the release of exudates.

2.10. **Induction of PLBs from different parts of plants (leaf / node/roots / shoot tip)**

Young leaves and leaf tips of orchids could be successfully cultured *in vitro*. The regeneration potential seems to be markedly influenced by the physiological age of the mother plant, position of the donor axis and growth-stimulus in the nutrient pool as has been suggested by Vajrabhaya, (1978). According to Vij and Pathak, (1990), leaf explants could be induced to regenerate *in vitro* by varying the chemical regime in various orchid species. Seeni and Latha, (2000), regenerated protocorm likes bodies from leaf base of 8 months old axenic seedling of *V. coerulea* on Mitra *et.al.* medium supplemented with 10 % CW, 500 mg/l peptone and a combination of BAP and NAA. Seeni and Latha, (1992), also cultured basal part of the young leaves of *Renenthera imscootiana* on M medium supplemented with peptone (2.0mg/l), BAP and NAA and obtained a large number of phenotypically uniform plants. Malabedi *et.al.*, (2004), reported efficient shoot regeneration of *V. coerulea* by using thin shoot tip sections on VW medium supplemented with 11.35 μM thidiazuron. Chaturvedi and Sharma, (1986), reported the protocorm formation from excised tips
of young leaves of *V.* hybrids. Mathew and Rao, (1985), succeeded in getting PLBs from the basal part of the leaf of *Vanda* hybrids.

Soediono, (1983), reported that by culturing shoot tip of *Dendrobium* in VW medium containing 15% coconut water plus 10 ppm NAA led to the rapid proliferation of PLB and plantlet formation as well as growth of the seedlings.

The application of shoot apex cultures for the clonal multiplication of plants was first realized by Morel, (1960), in his studies on the propagation of the orchid *Cymbidium*. Tanaka and Sakanishi, (1977), successfully demonstrated the formation of PLB’s and plantlets from young leaves of *Phalaenopsis* under influence of NAA and BA. Hass-von Schmude, (1984), reported that fully grown plants of *Phalaenopsis* could be obtained from meristems in 2-3 years, with the first flowering 4-6 months after removal from culture flasks.

*In vitro* root culture had so far been attempted with limited success in less than 10 species and hybrid of orchids. Goh, (1970), reported the development of thin etiolated structures from *in vitro* culture of root segment of *Vanda Miss joaquim*.

Chen *et al.*, (2004), reported plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum phillippinense*. Stewart and Button, (1975), reported that plantlets could be differentiated from a single *Paphiopedilum* stem apex, if bacterial free cultures could be obtained.

2.11. Cytological and karymorphological scenario of different taxa in context to the present study.

The study of chromosome behavior may play a pivotal role in the appropriate classification of orchids. Besides cytological study, it is a prerequisite for
hybridization programme. A sizeable number of workers kept themselves busy with the cytological studies since 17th century.

Chromosome behavior also helps the orchids taxonomists to arrange the orchid in appropriate order. The chromosomes derive their prominence as a tool in taxonomy from their direct relation to the genetic system of which is an integral part (Lewis, 1957). Senghas, (1975), stressed the use of cytogenetic data, microstructure analysis and numerical taxonomy in orchid classification.

The chromosome numbers are high in orchids. In the Indian orchids, the chromosome number range from $2n = 20$ (Cypripedium cardigerum, C. himalacium, Laris cordifolia) etc. to $2n = 164$ (Satyrim nepalense). The most common number, $2n = 38$, is represented in about 30% taxa. Other frequent numbers, $2n = 40$, 42, 30 are encountered in 24.8, 12.2 and 6.8 taxa respectively.

Among different cytological characters, karyotype study has been considered as one of the most reliable tools for identification and assessment of phylogenetic relationship (Naik, 1988). The morphological aspect of the chromosome complement as seen at mitotic metaphase or karyotype (Stebbins, 1971 ; Strickberger, 1995) is proved to be an important tool for identification of taxa (Kochhar, 1989; Power, 1990). The chromosome size and karyotype symmetry have served as good taxonomic characters and the absolute size of chromosomes in a karyotype is a fairly constant species-specific character (Devis and Heywood, 1961). Thus by comparing the karyotypes, the valid systematic position and phylogenetic relationship of closely related taxa can be established.
In recent past a number of cytologists have made phylogenetic assessment on the basis of karyotype analysis in different plant taxa. Shekhar and Vij, (1986), made cytotaxonomic studies in *Dendrobium* SW. and concluded that major speciation of Indian *Dendrobium* has revolved around the zygotic numbers \(2n = 38, 40\). Intraspecific coexistence of both these chromosome numbers in *D. amoenum* and *D. bicameratum* indicates their derivation from each other. \(X = 9, 10\) are considered as the primary basic numbers for *Dendrobium*. A total of about 250 species and varieties of *Dendrobium* have so far been cytologically investigated (cf. Tanaka and Kamemoto, 1974; Vij and Mehra, 1976). The majority of these show a somatic complement of \(2n = 38\). The other frequent chromosome numbers \(2n = 40\) and \(2n = 36\) in the genus are represented in 23 species and 7 species respectively. In some species of *Dendrobium* including *D. nobile*, the chromosomes are mostly median to submedian, whereas some subterminal and / or terminal chromosomes were observed in some taxa (Shekhar and Vij, 1986). Vij and Mehra, (1974), studied the cytology of east Himalayan *Orchidaceae* and found \(2n = 38\).

Biswas, (1986), studied the cytology of few cultivars of Indian Lady’s Slipper and found \(2n = 26\) in *Paphiopedilum villosum* and *P. insigne* and all the cultivars of *P. farrianum*. He also found \(2n = 41\) and 40 in *P. venustum* and its cultivars respectively. Accessory chromosomes were recorded in *P. insigne* cv. and *P. venustum*. Supernumerary constrictions were observed in *P. fairieanum* cv. *Giganteum*. Vij et. al., (1982), also studied the cytology of *P. insigne* and observed chromosome number as \(2n = 26\).
2.12. Production of artificial seeds

The concept of artificial seed was first coined by Murashige, (1977), at the symposium on tissue culture for horticultural purpose held at Belgium in 1977. He suggested the possibility of using this technique by covering embryods/meristemin in a nutritive gel and using them for propagation purpose. The production of artificial / synthetic / or somatic seeds/beads for the first time reported by Kitto and Janick, (1980), in carrot by using a mixture of carrot somatic embryos and callus with polyoxyethylene glyol. Since then, the technique found to be used extensively to several flowering plant species including orchids.

The production of synthetic seed was useful since orchids produce tiny and non-endospermic seeds. Several reports on encapsulation have been carried out using somatic embryos as the encapsulated propagules (Castillo et.al., 1998; Ara et.al., 1999). Reports on meristematic shoot tips or axillary buds for the production of synthetic seeds are also available (Ganapati et.al., 1992; Bapat et.al., 1987; Piccioni and Standardi, 1995). In orchids also, there are many reports on the production of synthetic seeds. Nayak et. al., (1998), in Spathoglottis plicata BL, Corrie and Tandon, (1991), in Cymbidium giganteum, Sharma et. al. (1992), in Dendrobium wardianum and Singh, (1991), in Vanda hokerriana. The production of synthetic seed was useful since orchids produce tiny and non-endospermic seeds.

2.13. Hardening and acclimatization of plants.

The hardening and acclimatization of in vitro raised plantlets is an important step in micropropagation for better survival and successful establishment of plants in ex vitro condition. The higher percentage of plant loss or damaged may occurred
during the transfer of *in vitro* raised plants to *ex vitro* condition. This is due to regenerates has to adjust to many abnormalities in *ex vitro* environment like high level of irradiance, low humidity and water hydraulic conductivity of roots and root-stem connections (Fila *et. al.*, 1998). Acclimatization of regenerates will overcome this treat with gradual lowering in air humidity (Bolar *et. al.*, 1998; Lavanya *et. al.*, 2009).

To acclimatize the micropropagated plants, various workers have employed different approach towards successful establishment of micropropagated plants in *ex vitro* condition. Sucrose concentration, agar in the medium is also said to have an effect on successful acclimatization to *ex vitro* condition (Hazarika, 2003; Synkova, 1997). Reducing the humidity level in the cultural vial also play an important role during acclimatization. Ziv *et. al.*, (1983), observed that reducing the humidity level inside the cultural vial has improved the internal structure of plants and give a more successful establishment in the glasshouse. Deb and Imchen, (2010), reported a novel and efficient one step hardening technique for tissue culture raised orchid seedlings. They used 1/10 strength MS (Murashige and Skoog, 1962), salt solution devoid of any plant growth regulators and sucrose or any other organic carbon sources. Alternatively, plantlets were also maintained with only tap water. The hardened plantlets of both epiphytic and terrestrial orchid from this new technique showed about 95% survival under poly house condition.

Bhargava *et. al.*, (2003), also found 92% survivability of *Phoenix dactylifera*, when plantlets were passed through pre-acclimation phase. Agnihotri *et. al.*, (2004), also reported 80% successful transplant of plants hardened in soilrite.