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# IN VITRO SEED GERMINATION AND PROTOCORM DEVELOPMENT IN TERRESTRIAL ORCHID *HABENARIA LONGICORNICULATA* J.GRAHAM FROM KOLHAPUR DISTRICT

**A. R. Patil and S. S. Patil**
Department of Botany, Rajaram College, Kolhapur 416 004, Maharashtra, India
patilsunita790@gmail.com

**Abstract:**
Orchidaceae represents a peak in the evolution of monocots and is one of the most successful families of flowering plants, as is clear from the wide distribution and its innumerable number of species spread all over the world (Arditti,1979).The innumerable number of dust like non- endospermous seed and their peculiar mode of germination which requires association with the mycorrhizal fungi are the peculiarities which characterise the orchid family. Orchid seeds are well known for their small size (0.15-6.0 mm) and their number per capsule from 6000 - 4 million and weighing as little as microgram (Ziegler,1981; Dressler,1993) In general they lack endosperm and instead form mycorrhizal relationship for germination and early growth. In present work attempt has been made to germinate orchid seeds in vitro by using a tissue culture media (Knudson C). Seeds of terrestrial orchid *Habenaria longicorniculata* J. Graham were cultured in an attempt to raise the seedlings up to the level of protocorm development.

**Keywords** : Orchids , *Habenaria longicorniculata*, mycorrhizal fungi ,Knudson C media.

**Introduction :**

Orchidaceae represents a peak in the evolution of monocot and is one of the most successful Families of flowering plants, as is clear from the wide distribution and its innumerable number of species spread all over the world.(Arditti 1979).The innumerable number of dust like non endospermous seed and their peculiar mode of germination which reqires association with the mycorrhizal fungi are the peculiarities which characterise the orchid family. Orchid seeds are well known for their small size (0.15-6.0 mm) and their number per capsule from 6000-4 million and weighting as little as microgram (Ziegler 1981; Dressler 1993) In general they lack endosperm and instead form mycorrhizal relationship for germination and early growth. The fungi supplies young orchid sampling with the required sugar and nutrients until orchid plants are large enough to produce their own food. Once seed has germinated it produces a fairly undifferentiated mass of cells called a protocorm. In terrestrial orchids it is important that orchid fungus relationship is maintained during early stages of plants life, as protocorm is subterranean and can- not produce any food of its own. In the present work, attempt has been made to germinate orchid seeds in vitro by using a tissue culture media (Knudson C). For the study we have used seeds of terrestrial orchid *Habenaria longicorniculata* J.Graham and raised the seedlings up to the level of protocorm development.

**Material and Methods :**

Survey and collection of *Habenaria longicorniculata* J. Graham was conducted during the rainy season from various parts of Kolhapur district. Mature capsules were collected in clean bottles separately and dried. A few entire plants with tubers before flowering were collected and planted in the botanical garden in the college. After flowering, the flowers were artificially pollinated and then capsules were collected and dried. Seed morphometric studies were carried out using research microscope and SEM. Viability test for seeds was carried out by using TTC. Media Knudson C, Orchid Agar and MS media were prepared supplemented with auxin and used for seed culture.

**Results:**

**A] External Morphology of *Habenaria longicorniculata* J. Graham**

Terrestrial herb, 2 -3 feet in height. Tubers 1 - 2, ellipsoid. Leaves 3 - 6, clustered at the base of the stem, oblong-lanceolate, base narrow, sheathing, acute. Inflorescence raceme. Flowers white, jasmine-scented, pedicelate; pedicels up to 4 cm long, pale green, ribbed, bracteate, at the top of long, slender peduncle; floral bracts cover the ovary, bracts oval to oblong-lanceolate, acuminate. Sepals 3, white with green tinge. Petals white with green tinge, oblong-lanceolate, with trilobed lip. Lip 3-partite, margins of lobes dentate. Spur slender, pale green, up to 10 - 15 cm long. Ovary curved, with strong ribs, beak long. Capsules long, narrowly fusiform, beaked.

Flowering and Fruiting: August - September.

Localities: Kolhapur, Pune, Satara, Amboli, Mumbai, Karnataka (Figure 1).

**B] Localities of collection** (Table 1)

**C] Capsule :** Capsules of *Habenaria longicorniculata* J. Graham were collected from the mature orchid plants grown in Botanical Garden of Rajaram College Kolhapur and collected from the field were used as the source of plant materials. The capsule donating orchid plants were grown in natural field conditions after flowering, several flowers were hand pollinated on the second of anthesis. The pollinated flowers were bagged with butter paper for one week. Several capsules of *Habenaria longicorniculata* J. Graham were harvested 120 days after pollination and brought to the laboratory for *in vitro* seed germination.The capsules were surface sterilized by submerging them in a 0.2% (w/v ) sodium hypochlorite (NaOCl) solution for 10 minutes and were washed 3-4 times with distilled autoclaved water under a laminar flow hood and then used for further investigation.

**D] Seed morphology and viability studies :**

The seed colour ranges from off white pale yellow and pale brown to dark brown.Testa cells are usually transparent, with outer smooth or reticulate cell walls.The light microscopic observation as well as SEM study also carried out to study the seed shape ,colour ,embryo volume and colour of the testa. For seed viability analysis, freshly isolated seeds from capsules were stained with 1% (v/v) 2, 3, 5-triphenyl tetrazolium chloride (TTC) in darkness overnight under 24 h. The seeds were then observed under a stereomicroscope. Percentage of seed viability was calculated by the number of red colour staining embryos divided by the total number of seeds examined and multiplied by 100 (Figure 2).

**E] In vitro seed germination : (For Asymbiotic Seed Germination.)**

Capsules of *Habenaria longicorniculata* J. Graham were used as seed explants. The capsules were disinfected and cut vertically over a sterile Petri-dish. The seeds were then removed and placed on the filter paper. The seeds were surface sterilized again by dipping into 15% (v/v) sodium hypochlorite (NaOCl) solution containing rinsed three times with sterile distilled water, and placed on sterile filter paper to blote them. Three different basal media were investigated for germination of seeds. These were MS (Murashige and Skoog, 1962) Knudson C (Morel Modification) and Orchid Agar. All of these media were supplemented with auxin in different concentrations and Seeds were germinated for several weeks in a growth chamber maintaining a temperature regime of 25±2°C, 88% relative humidity. The pH of all plant culture media was adjusted to 5.8. All media were autoclaved at 121°C for 20 minutes under a pressure of 15 lbs/sq (Figure 3).

**(For Symbiotic Seed Germination.)**

In symbiotic seed germination the seeds are sown with a small piece of an appropriate mycorrhizal fungus.The fungal culture is freshly prepared and dissolved in distilled water. This fungus colonise the germinating seeds and a symbiotic relationship is formed which sustain the protocorm and thire subsequent development. The procedure is same like asymbiotic seed germination the only difference is fungal inoculation along with seeds (Figure 4).

**Table 1:** Localities of collection

| Sr. No | Name or Orchid | Date | Place | Date | Place | Date | Place |
|---|---|---|---|---|---|---|---|
| 1 | *Habenaria longicorniculata* J. Graham | Aug.-Sept. | Vaibhavwadi | 30/08/13 | Amba | 17/07/14 | Radhanagari |
| | | 12/09/12 | Amba | 14/09/13 | Tillari, Chandgad | 19/08/14 | Kas, Satara Bamnoli, Thoseghar |
| | | 08/09/12-09/09/12 | Malshej ghat, Bhimashankar, Vichitragad | 15/07/13 | Radhanagari | 07/09/14 | Patgaon, Pal |
| | | Aug.-Sept. | Tillari | 16/08/13-17/08/13 | Malshej, Vichitragad | 15/08/14-18/08/14 | Uran, Malshej |
| | | 05/09/12 | Gawase Devrai, Ajara | 08/10/13 | Satara, Kas, Bamnoli, Thoseghar | 10/08/14 | Patgaon, Pal, Bhatwadi |
| | | Sept 2012 | Matheran, Uran, Lonavala | Sept.2013 | Amboli Choukul | 03/08/14 | Amba |
| | | Aug.- Oct. | Karanj, Mumbai | 08/11/13 | Bugate-Alur | 29/07/14 | Ajara, Tillari |
| | | Aug.- Oct. | Bugate-Alur | 16/08/13-17/08/13 | Bhimashankar | 29/07/14 | Kasar Kandgaon |
| | | 08/09/12-09/09/12 | Malshej ghat, Bhimashankar, Vichitragad | | | 29/07/14 | Ajara, Tillari |

**Table 2**

| Sr. No. | Culture Media | Protocorm Formation | Temperature | Relative Humidity |
|---|---|---|---|---|
| 1. | Knudson C (Morel Modification) | √ | 20 - 25$^0$ C | 88% |
| 2. | Orchid Agar | × | 20 - 25$^0$ C | 88% |
| 3. | Murashige and Skoog | × | 20 - 25$^0$ C | 88% |


**Figure 1**

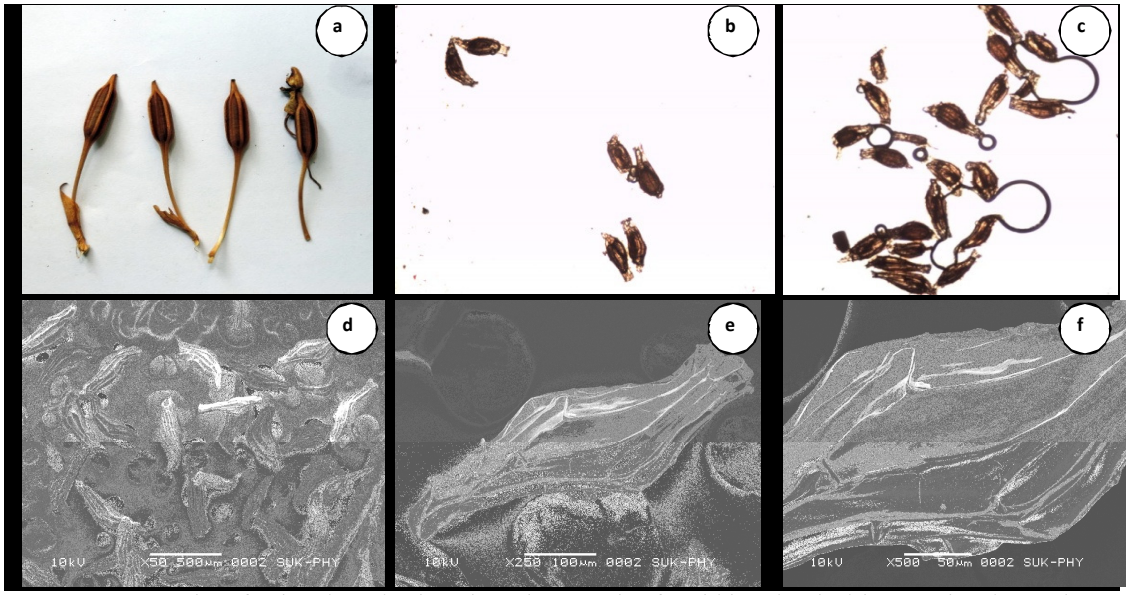


**Figure 2** a- Capsules of H.longicorniculata, b and c – seeds of orchid stained with TTC showing embryo, d,e ,f – SEM Microphotograph of seeds showing seed structure and testa.

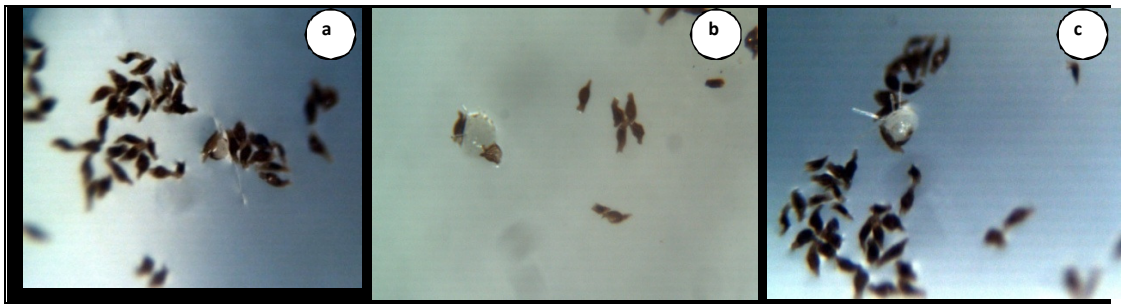


**Figure 3** a , b , and c showing ruptured seeds of orchid and development of protocorm

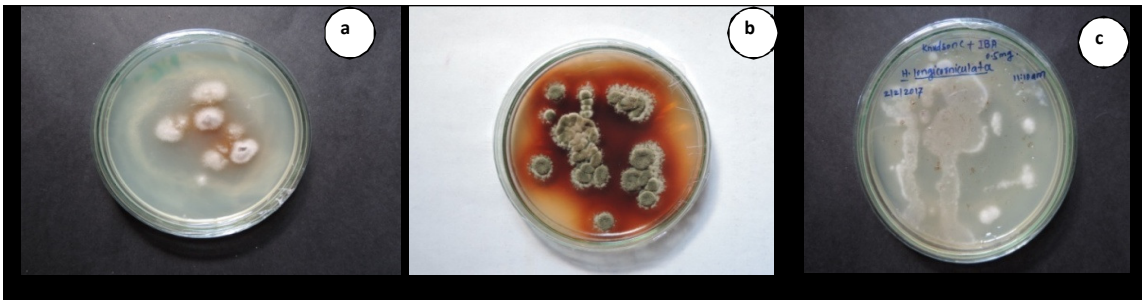


**Figure 4** a. and b. Pure culture of mycorrhizal fungi on PDA. c.Orchid seeds cultured with mycorrhizal fungi on culture media.

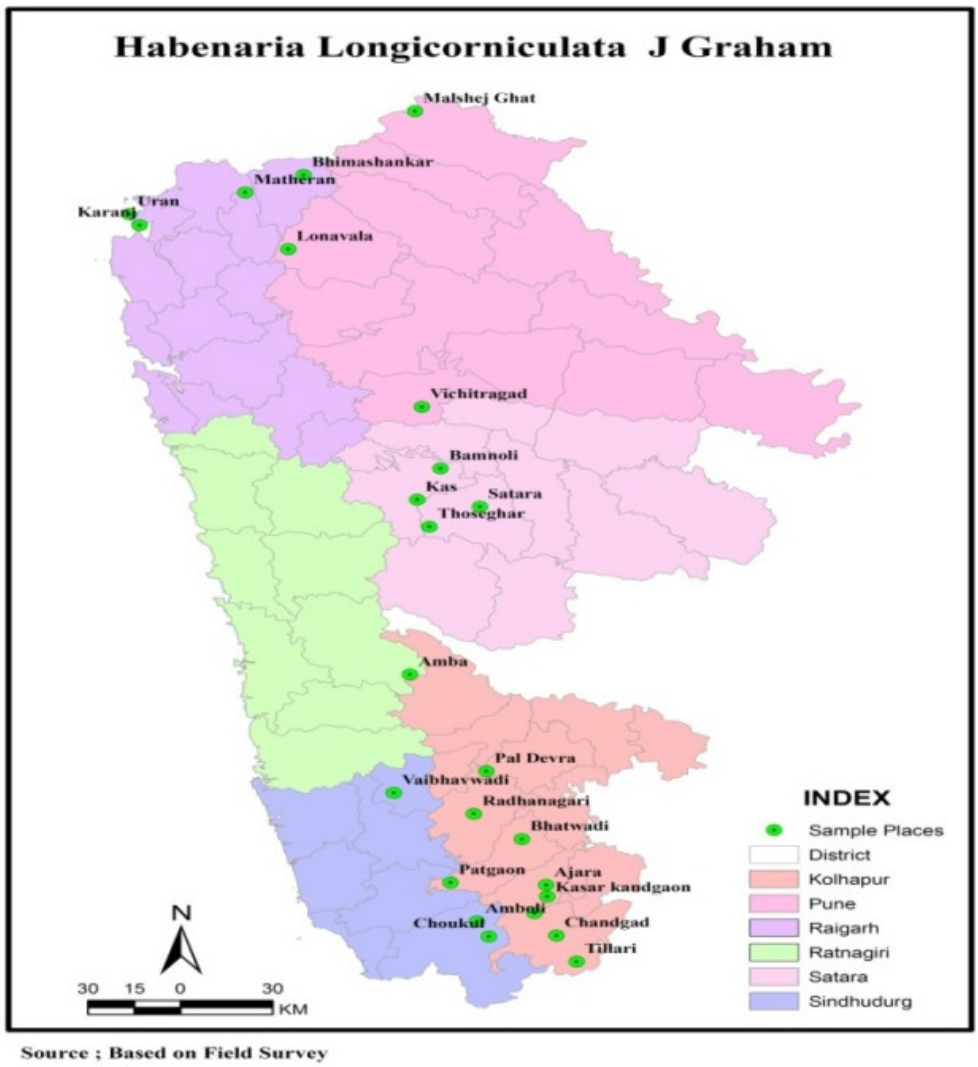


**Figure 5** Location Map : *Habenaria longicorniculata* J. Graham

**Discussion**

The seeds taken from the mature capsules were sown on the different medium containing various concentrations of plant growth regulators. Due to the non-endospermic nature of the seed, the germination in nature is a unique phenomenon and requires fungal infection. Germination is much more successful in *in vitro*. The production of orchid seedling from seed involves, sequential phases of germination, protocorm formation and seedling development. In the present investigation also same sequence of seedling development was observed when the selected orchid, *H. longicorniculata* was grown on the culture media, as the embryos development into globouse protocorms, seed coat

(testa) got ruptured and rhizoids were getting formed. Among the three different culture media ,Knudson C (Morel Modification ) was found to be the most suitable, which supported germinations of seedlings and formation of protocorm like bodies.In case of symbiotic seed germination protocorm development was not found (Table 2).

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