RESEARCH COMMUNICATIONS

The theory of linear estimation of regression parameters is being extensively used owing to its computational convenience. However, the computational simplicity that comes with the linear model is often lost when one is faced with obtaining point and interval estimates for the original rather than the transformed parameters. For example, say, when the emphasis is on estimating the expected longevity ($L$) for a given moisture ($m$) and temperature ($t$), simply de-transforming the usual least squares prediction equation can lead to severe bias. Linear model estimation gives us the estimate of Log($L$). Thus, it is the naive estimate of longevity that we estimate by taking antilog of estimate of Log($L$). If errors of Log($L$) are symmetric then errors of $L$ are asymmetric and thus, estimate of $L$ tends to estimate the median rather than the mean of the distribution of $L$. However, before adopting the nonlinear estimation procedure for other species, it would be worth studying the behaviour of the model under experimental conditions and validating the model for extrapolated conditions. For validating the model one may generate two sets of data. The first set may be used for estimating the parameters and the other for validating the model. Utmost attention should be paid to estimate the parameters with high degree of precision. If the linearizing transformation is not successful, particularly in stabilizing the error variance, one should not go for the ordinary linear least square estimation procedure. Estimates with low precision should not be released as they may prove more damaging. Trusting on such estimates may ultimately lead to a loss of valuable genetic wealth stored for long-term genetic conservation.


Use of image analysis to study the effect of phosphate on honeydew formation and clavine alkaloid synthesis in *in vitro* cultures of *Claviceps fusiformis* Lov.

Asha Jacob* and Paramjeet Jite
Department of Botany, University of Pune, Pune 411 007, India

Image analysis has become an important tool in the study of growth and nutrition in fungi. Here this technique has been used successfully to study the effect of phosphate on honeydew formation and clavine alkaloid synthesis in *in vitro* cultures of *Claviceps fusiformis* Lov. Specific colour reagents for the detection of clavine alkaloids and phosphate are used to demarcate the region of alkaloid production and the pattern of phosphate utilization by the *C. fusiformis* colony. The intensity of colour formed is then measured using image-analysis techniques, and regions of similar intensity are coloured alike using pseudocolours. This enables the division of the colony according to its biochemical make-up, to an accuracy not achieved by the naked eye. Growth of the colony can also be monitored nondestructively on a daily basis. It is seen that as the colony grows, phosphate in the medium is depleted, disrupting growth and causing a decrease in intracellular phosphate. This leads to honeydew formation accompanied by enhanced clavine alkaloid production.

*CLAVICEPS fusiformis* Lov. causes ergot, a commonly occurring disease on members of Gramineae. Ergot alkaloids cause ergotism in men and cattle on consumption, but have gained importance owing to their pharmacological properties. These clavine alkaloids are also used as a precursor in the synthesis of lysergic acid diethylamide and LSD. Parasitic cultivation of this fungus besides bearing the risk of poisoning, also demands large areas of cultivation and consequent loss of food crop. Therefore, fermentative cultivation of this fungus where the process can be precisely controlled, is a welcome alternative. To optimize nutritional conditions that would maximize biomass and alkaloid production, it is important to raise *in vitro* honeydew stage, as the latter marks the advent of secondary metabolism and the production of alkaloids. *In vivo* honeydew formation requires entry of long filamentous hyphae raised from germinating ascospore of *C. fusiformis* through the stigma and into the ovary of the flower. These come in contact with the epidermal tissue at the base of the ovary and cause plasmolysis. The infected hyphae feeds on the host sucrose and converts it into fructose and glucose by the action of $\beta$-D-fructo-

---

*For correspondence. (e-mail: ashajacob2000@hotmail.com)*

1616

CURRENT SCIENCE, VOL. 85, NO. 11, 10 DECEMBER 2003
furanosidase and β-D-transfructofuranosidase, causing sucrose deficiency in the tissue. This causes further flow of plant sap into the infected area, furnishing the parasite with nutrients for its growth. The interwoven hyphal mass increases in thickness and occupies the entire ovarian cavity, kills the floret and forms the honeydew stage. Phosphate is the other essential compound needed for its growth. The pyrophosphates are used for the generation of ATP, while the orthophosphates are required for the generation of proteins and nucleic acids. This communication attempts to bring out the effect of phosphate on honeydew formation and subsequent alkaloid synthesis, both of which are seen to be triggered through depletion of this element in the culture medium.

Pure cultures of *C. fusiformis* were raised on modified CNM medium and Kirchoff’s medium from sclerotia formed on *Pennisetum typhoides*. The cultures were grown at 20°C and in the dark. Mycelial strands started to appear after 5–6 days and the culture was ready for subculturing within 8–9 days. Plugs of 5 mm diameter were cut from the margins of actively-growing colonies and inoculated on petri plates containing Kirchoff’s medium covered with washed and autoclaved cellophane discs. After three weeks of growth, the mycelium at 18°C shows one or two colourless droplets, which increased in number and size with age (Figure 1a). These droplets turned pink to dark brown after about three months. This formed the honeydew stage (Figure 1b). On microscopic observation these showed the presence of conidiophores and fusiform conidia as found under *in vivo* conditions (Figure 1c). However, even on prolonged incubation the honeydew did not develop into sclerotia; on drying it formed a black-coloured hard mass which did not contain any ascospores, but showed conidiophores. The cellophane was

![Figure 1](image-url)

Figure 1. *a*, Two-week-old *C. fusiformis* colony showing minute droplets of honeydew at the centre. *b*, Honeydew formed in three-month-old culture. *c*, Conidiophores showing the formation of fusiform conidia as seen under a microscope (magnification 40X × 10X). *d*, Test plate showing phosphate utilization by the colony. *e*, Fungal colony showing the region of clavine alkaloid synthesis. (*f–h*) are pseudocolours of (*a*), (*d*) and (*e*). (*e*) shows the fungal mycelium with an intensity of white at 232, which is seen as blue in (*f*), while the pink droplets of honeydew are seen as an area of 225 and shown as light blue in the pseudocolour in (*f*). (*d*) shows an intensity of black being 63 in the unused medium, the centre blue has an intensity of 98, while the centre light-coloured ring has an intensity of 180 (pink shown by the green colour in (*g*)). (*e*) shows a central dark blue of intensity 39 (the central blue colour in the pseudocolour (*h*)), followed by a lighter blue of 140 (pink zone in (*h*)). The actively-growing region in the mycelium showed no alkaloid production and an intensity of 210 in (*e*). This was seen as green colour in (*h*).
lifted-off with the colony and was placed with the cellophane-side facing up in a clean petri plate. The petri plate was then filled with modified Van-Ureks reagent to submerge the cellophane and incubated for 10–15 min. The nitrogen in the indole ring of the alkaloid reacts with sulpharic acid and ferric chloride of the reagent and forms a blue complex. The distribution of this colour indicated the region of production of the alkaloid in the colony (Figure 1c). The phosphate detection reagents described by Trivedy and Goel, was added to the test plates from which the colony of *C. fusiformis* had been removed. This was incubated for 3 h to enable the colour to develop, after which the reagent was drained-off. Phosphate in the test plate reacted with ammonium molybdate and formed a complex heteropoly acid, molybdophosphoric acid which gets reduced to form a blue colour in the presence of stannous chloride. The distribution and intensity of blue colour in the plate indicated phosphate utilisation by the fungal colony (Figure 1d). Well-designed staging of the specimen was required, with adequate and uniform illumination. A video camera was used to capture the images which were digitized and analysed using the imaging software WG-DejaVu. The images thus captured comprised of a pixel array that showed the morphometric (spatial) and photometric (spectral) features of the specimen. Each of these pixels is assigned a numeric

![Figure 2](image_url)  
**Figure 2.** Relation between (a) phosphate metabolism and growth, (b) alkaloid production and growth.
value which shows the intensity of white or black. White has an intensity of 250 and black, 0. Therefore, all the colours would fall in a range between these two values. This would also mean that a lower numeric value would show greater pigmentation. Accordingly, regions having the same intensity are given the same colour or pseudocolour (Figure 1f–h). Therefore, image analysis of tissue treated with different reagents enabled division of the tissue according to its biochemical make-up.

Growth of the colony was measured on a daily basis by measuring its diameter. The individual colonies of *C. fusiformis* grown in solid phase are more or less circular (because of the inoculation of fungal discs). Using WGDeyaVu software the diameter of each of these colonies is then measured twice at 90° to each other, daily. The average of these diameters expressed as cm/day gives the growth rate. On comparing the test plates with the pseudocolours it was observed that the unused medium has maximum phosphate followed by the centre zone under the old tissue, as evident from Figure 1. Least amount of phosphate is seen in the region under the growing mycelium. Phosphate metabolism and total alkaloid production in the region of the oldest mycelium (the centre of the colony) were also monitored from the fifth to the twentieth day, as shown in Figure 2a and b. The growth phase of the colony was seen to continue till the ninth day, after which the colony enters the stationary phase where there is a decrease in growth and intracellular phosphate with the advent of secondary metabolism. Since colour intensity is measured as a decrease in white intensity, the graphs show a downward trend on production of increased alkaloid and non-utilization or expulsion of phosphate. Therefore, a downward trend of alkaloid or phosphate in Figure 2a–c would actually mean an increase of the same and vice versa.

Using image analysis a nondestructive and indirect online measurement of growth is possible. It is evident that the biosynthesis of honeydew in *vitro* requires changes in metabolism and a decrease in mycelial growth. This was seen when there was no honeydew formation during the active growing period, and is formed as an alternative measure taken by the fungus to compete with a depletion in nutrients accompanied by a decrease in growth. The enzymes responsible for honeydew formation are repressed or inhibited under conditions of rapid growth, or are present in their latent form in mycelia from high-phosphate medium, or actively-growing colony. As seen from image analysis, the growing tip of the colony requires large quantities of phosphate. This was seen when the medium corresponding to the growing tips did not take up any colour, indicating a depletion of phosphate in the medium. The decrease in intracellular phosphate was largely visible at the centre of the colony or the region of the oldest tissue, which also happens to be the region of honeydew formation. This is evident by the blue colour developed on the agar plate. Therefore, there is a utilization of phosphate by the growing tissue and the expulsion of the same by the older tissue. This is also the area of maximum alkaloid synthesis. Image analysis** has proved to be an important tool in determining the exact nature of this relation.

The honeydew formed *in vitro* did not show significant level of secondary metabolism, but it is obvious that this stage can be obtained *in vitro* without the host, whereas the development of honeydew into sclerotia needs the ovary of the host. Therefore, mimicking the factors favour-
ing its formation could spell the success of a two-phase culture for optimum growth and maximum production in *C. fusiformis* mycelium and the economic extraction of the clavine alkaloid.


ACKNOWLEDGEMENTS. We thank Dr R. V. Gadre, Chemical Engineering Division, National Chemical Laboratory, Pune for critically reading this manuscript. We also thank V. Udapikar and P. Mathapatil, Wavelit, Pune for help with image analysis.

Received 1 May 2003; revised accepted 16 August 2003

**Ecology of microsporangium dehiscence and pollen flow in Himalayan long-needle pine (Pinus roxburghii Sargent)**

**V. P. Khanduri and C. M. Sharma**

Department of Forestry, HNB Garhwal University, Post Box 76, Srinagar Garhwal 246 174, India

Effect of temperature and relative humidity on microsporangium dehiscence and receptivity of megasporophylls in *Pinus roxburghii* were analysed. Ovulate strobili remained receptive up to five days with optimum receptivity between 1200 and 1600 h.

POLLEN is an effective process of transfer by pollen vectors from the male to the female flower/organ, dependent primarily on air temperature and humidity. Wind is an important pollen vector and efficiency of wind pollination is generally believed to decrease as the concentration of airborne pollen decreases.1,2 This low efficiency may be due to large distances between conspecifics, low pollen production by individuals, poor pollen dispersal and wind velocity. These conditions are assumed to affect both ovule fertilization and subsequent seed production, negatively.

*Pinus roxburghii* Sargent (Chir pine or Himalayan long-needle pine), a valuable timber-resin tree of the Central–western Himalayan region is also a wind-pollinated species, but little is known about its reproductive biology, particularly pollination ecology. Therefore, the present study was initiated to understand microsporangium protrusion, and pollen liberation and pollen flow in *P. roxburghii* under different weather conditions.

This study was conducted with five representative trees (ten strobili per tree), in the natural populations of *P. roxburghii* at Ashtavakra (900 m amsl) in the Pauri Garhwal district, situated between 29°20' and 30°15'N lat and 78°10' and 79°20'E long.

Randomly selected strobili in the crown were examined at 1 h intervals for entire daylengths. Microsporangium dehiscence was recorded by the scoring and removing method, to avoid duplication, with the help of a hand lens (× 20). Prevailing air temperature, relative humidity and wind speed were also recorded each time close to the strobili. Observations were recorded on six different days under different weather conditions: clear sky with heavy dew and little dew; cloudy with no dew and no rain; cloudy with heavy rain and drizzling, and partially cloudy, to decipher exactly the effect of weather factors on microsporangium protrusion.

Pollen concentrations in the atmosphere were observed on jelly-coated microscopic slides, mounted vertically on iron rods and placed perpendicular to the direction of prevailing wind or all around the source tree, viz. north, south, east and west directions. The rods were mounted on the trees at heights corresponding with those of the pollen cones, and the slides were replaced at every 2 h intervals. The number of pollen grains per slide was counted under the binocular microscope on an area of 1 cm² (1 cm × 1 cm). Twenty slides were placed around each source tree (five slides per direction). The distance of each slide from the source tree was approximately 5 m. This experiment was performed on three different source trees. Similarly, megasporophylls (stigmas) on ovulate strobili were used to observe pollen concentration and/or the rate of pollen deposition at every 2 h intervals, to decipher the best time of the day for pollination. The stand density of reproductively matured male individuals was 160 trees per hectare. The ovulate strobili were exposed to wind in batches for desired intervals up to three days. They were then removed from the source tree and placed in plastic jars with cotton wadding saturated with formalin-acetato-alcohol, according to Ornduff.

The effect of disturbance on pollen concentration was verified by subjecting the physical disturbance to the bloomed bough under undisturbed conditions inside a room by arranging jelly-coated slides at 1 m distance all around, on the same level. The rate of release of pollen grains into the ambient air was also verified by moving a

*For correspondence. (e-mail: vkhanduri@yahoo.co.in)*

CURRENT SCIENCE, VOL. 85, NO. 11, 10 DECEMBER 2003