CHAPTER IV

DISCUSSION

C H A P T E R   I V

D I S C U S S I O N

Voluminous work has been carried out on ergot alkaloids in Claviceps sps and other related organisms. The presence of these alkaloids in few higher plants belonging to the family Convolvulaceae has been reported. But as mentioned in the Introduction (Chapter I) no detailed work has been carried out beyond screening a few plants. It was also found that intact higher plants contained very less amount of ergot alkaloids compared to the ergot fungus, Claviceps sps.

The culture of angiosperm cells or tissues derived from the intact plant body, have now become widely exploited as an experimental tool. Recent advances in tissue culture techniques have made it possible to investigate factors controlling the pathways of secondary metabolites. Various reports have also indicated that alkaloid production could be enhanced by adjusting the nutritional requirements, by manipulating the levels of hormones, as well as by feeding with known precursors.

In light of this information, different experiments were carried out on Evolvulus alsinoides; a common weed of known medicinal value. Results described in Chapter III present finding on the "In vivo and In vitro studies on growth and ergot alkaloid production in E. alsinoides". The data emphasise the significance of such work to open new vistas for ergot

alkaloid production in angiosperms which were not carried out until now.

The present study was undertaken with the following objectives as outlined in the Introduction (Chapter I) : (i) To analyse the system for most rapid growth and alkaloid production, (ii) To explore the factors regulating the alkaloid biosynthesis, (iii) To study the known enzymes controlling the production of ergot alkaloids and, finally (iv) To find out the correlations, if any, between the microorganisms and higher plants in the biosynthesis of ergot alkaloids.

The experimental materials used for this purpose were :- (i) Intact plants of Evolvulus alsinoides L. for "In vivo" studies.

(ii) Tissue cultures initiated from young leaves of E. alsinoides.

The results of the experiments conducted to realise above stated objectives are described in the earlier Results (Chapter III) section of the thesis. To what extent the evidences obtained in the present investigations have realised the aims and objectives, now remains to be assessed.

The results obtained in this pursuit are discussed in the present chapter, with reference to other reports under the following captions :

I - "In vivo" studies on alkaloid production in Evolvulus alsinoides.

II - "In vitro" studies on growth and alkaloid production in E. alsinoides :

(A) Effect of cultural parameters on growth of tissue.

(B) Effect of cultural parameters on alkaloid production and the activity of Tryptophan synthetase.

Section - I   "In vivo" Studies on alkaloid production in Evolvulus alsinoides L.

The results in Section A of Chapter III, clearly indicated that the alkaloids are accumulated in maximum quantity in the seeds. The percentage of alkaloids was more in seeds; whereas older leaves, stem and flowers did not show the presence of these alkaloids. Young leaves also showed the presence of alkaloids, but in comparatively smaller quantities; whereas roots and young seedlings had a comparatively higher quantity of alkaloids. As mentioned in the introduction the site where maximum content of alkaloids is found may not be the actual site of its synthesis. Mockaites, et al. (1973) have observed in Ipomoea species, that ergot alkaloids are synthesised in leaves and Gröeger (1963) have found that the alkaloids are accumulated in the seeds which themselves cannot synthesise them. The evidence obtained here seemed to indicate that it is in the

young leaves that the alkaloids are synthesised from where
they are translocated to the roots where the alkaloids are
stored.

Section - II. (A) Effects of cultural parameters on growth
                of tissue in E. alsinoides suspension cultures.

Evolvulus leaf callus was originally initiated in MS
medium supplemented with 2.0 mg/l 2,4-D and 0.4 mg/l kinetin
in addition to 2% sucrose. The callus tissue was transferred to
different auxin and cytokinin levels separately and in combinations
for finding out optimal hormonal supplement that would support
rapid growth of tissue. Among the various auxins tried; 2,4-D
at 2 ppm, NAA at 5 ppm and IBA at 0.5 ppm, induced healthy
growth of tissue (Chapter III, Section C-1). On the contrary,
GA_3 at the different concentrations tested in the medium did not
promote growth of tissue compared to others (Section C-3). The
results showed that the auxins supplied exogenously promoted
growth at lower concentrations, whereas growth was suppressed
at higher levels of auxin, suggesting that the regulatory
mechanism of auxin was controlled by its levels maintained
endogenously.

The only cytokinin examined (Chapter III, Section C-2)
was kinetin. Kinetin supported the maximum growth till 0.4 mg/l
concentration whereas higher concentrations had inhibitory
effects. Similar inhibitory results by high levels of kinetin

had been observed in cell suspensions of <u>Acer</u> <u>pseudoplantanus</u> L.
(Simpkins and Street, 1970).

GA_3 (Chapter III, Section C-3) at all the concentrations
tried, supported less growth of tissue; increasing concentrations
of GA_3 being inhibitory to tissue growth. Nickell and Tulecke
(1959) have reported that growth inhibition was the most common
tissue response to the incorporation of gibberellic acid in the
nutrient medium. Further, Straus and Epp (1960) have showed that
use of different auxins in conjunction with Gibberellins did not
change the response appreciably. Paleg (1961) has explained that
possibly the release of protein nitrogen induced by GA caused
loss in cell dry weight. The inhibitory effect of GA_3 on growth
of other tissues has also been reported (Murashige and Skoog,
1964, 1965).

Further, (Chapter III, Section C-4) the combination of
various auxins and GA_3 with 0.4 mg/l kinetin was studied. Among
the various combinations tried, 2 mg/l 2,4-D with 0.4 mg/l
kinetin gave maximum effect on growth. Similar observation has
been made with <u>Ipomoea</u> <u>biloba</u> (Bhatt, 1977). The requirement of
high auxin response was clearly shown by Murashige and Skoog
(1962) and Khan (1975), where they have suggested that kinetin
might antagonise an inhibitor, thus allowing auxin to function
most efficiently. The present studies further confirm Auxin-
Kinetin synergistic effects on growth.

Comparative studies between static and suspension cultures

(Chapter III, Section C-5) clearly showed that growth of tissue as well as alkaloid production was appreciably higher in liquid shake cultures. This might be on account of efficient uptake of nutrients by call suspensions. Similarly, an increase in dry weight and nitrogen content in suspension cultures compared to static cultures has been reported earlier in Arachis hypogaea (Shailaja Rao, 1979).

Experiments on inoculum size/medium volume ratio (Chapter III, Section D) clearly indicated that 200 mg tissue by fresh weight in 25 ml of medium gave higher growth compared to other treatments. Rao (1971), while studying the effect of inoculum size/volume of medium in Datura cultures, reported that 100 mg tissue by fresh weight in 60 ml of medium registered maximum growth among the combinations tried. Henshaw et al. (1966) had shown that in a fixed volume of the medium, the highest growth occurred when the inoculum is low, and by increasing the inoculum size there is gradual decline in growth of tissue, indicating that the growth was limited by the supply of an essential nutrient in the medium. The results in the present studies also clearly support that inoculum size/volume of medium regulated the final yield of tissue.

Superiority of sucrose as carbon and energy source for the majority of plant tissues grown in culture is well recognised as stated in the Introduction. Present studies with Evolvulus alsinoides L. suspension cultures (Chapter III, Section E-1 & 2) also showed that sucrose is a better carbon source than the

other sugars studied. Glucose and Fructose alone or in
equimolar combination did not enhance the growth of tissue as
sucrose did. Further, sucrose at 2% responded maximum growth;
higher concentrations as well as lower concentrations were
less effective. Rao (1971) had also reported the superiority
of sucrose over other sugars as carbon and energy source in
Datura tissue cultures.

Studies on nitrogen requirement (Chapter III, Section-F)
showed that the standard culture medium containing both
ammonium and potassium nitrate as nitrogen sources, formulated
by Murashige and Skoog (MS medium, Chapter II) supported maximum
growth of tissue than any other concentrations tried. Higher and
lower levels of total nitrogen studied had no promotory effect
on growth. Studies carried out with carrot and potato cell
cultures (Steward et al., 1958; Shantz and Steward, 1959) and
with Cucumis (Fadia, 1971) had also revealed that suitable
combinations of calcium and potassium nitrates with ammonium
nitrate were optimal for growth. On the contrary, studies with
tobacco (Filner, 1965), soybean (Gamborg and Shyluk, 1970) and
Benincasa (Bhatt et al., 1973-74) cells have demonstrated that
ammonium nitrate alone was more suitable over other inorganic
nitrates for their growth.

Among the various combinations of microelements and macro-
elements tried (Chapter III, Section-G), increased concentrations
of macroelements had profound effect on growth, doubling the

levels of micro and macroelement salts recording the maximum growth. The results also indicated that microelements are essential for active growth of tissue because in the absence of microelements, the growth of tissue was much less compared to the control, recording a fresh weight increase of 12 fold and dry weight increase of 10 fold compared to 17 fold and 13 fold increases respectively in control. Similarly, the role of macro-elements for growth of tissue was clearly indicated in the study of different levels of microelements in absence of macroelement. In these cases the tissue hardly showed any growth. Increasing the level of microelements alone did not enhance growth, which indicated that macroelements also played prominent role in tissue growth of Evolvulus alsinoides L.

Palmitic acid (Tween-40), Stearic acid (Tween-60) and Oleic acid (Tween-80) are the surface reactants commonly known as Tweens. Rao and Satya Rao (1975) have shown that in the presence of Tweens in the medium, the medium components were utilized by Aspergillus fumigatus at a higher rate than in the untreated control. It was also noticed that Tween-60 and Tween-80 helped in the higher utilization of glucose and ammonia. However, in the present studies (Chapter III, Section-H) addition of Tweens had an adverse effect on growth of tissue. This might be due to their inhibitory effect on primary plant metabolism. The increased alkaloid production seemed to indicate that higher level of Tryptophan in the tissue was directed towards alkaloid production and not to other primary metabolic reactions resulting in less growth of the tissue.

Various precursors tried mainly for study on alkaloid
production were L-tryptophan, Mevalonic acid, Anthranilic acid,
Indole, Serine and Methionine. All the above precursors except
Mevalonic acid (Chapter III, Section-I) had an adverse effect
on growth of tissue. Tryptophan at higher levels, Indole at all
levels tested, Anthranilic acid and Methionine at all levels
tested completely inhibited the growth of tissue, the tissue
becoming brownish in colour and later senesced.

There are sufficient reports regarding the inhibitory
effects of various amino acids on tissue growth. Behrend and
Mateles (1975) have reported that certain amino acids inhibit
growth of tobacco, tomato, carrot and soybean cell cultures when
nitrate or urea are the nitrogen sources but not when ammonia
was the nitrogen source. These amino acids were found to inhibit
the development of nitrate reductase in tobacco and tomato cell
suspensions. Similar observations were made by Rao (1971) in
Datura cell suspensions. This suggested that amino acids
inhibited assimilation of intracellular ammonia into amino acids
in cells grown on nitrate and urea. Further, Radin (1975) has
also reported that Glycine, Glutamine and Aspargine strongly
inhibited induction of activity by nitrate and also decreased
growth of sterile - cultured roots on a nitrate medium. Methionine,
Serine and Alanine were also found to inhibit growth induction.
The present studies with different amino acids also recorded a
similar pattern in growth of tissue, where ammonium nitrate and
potassium nitrate were the nitrogen sources. This suggested that

the amino acids incorporated must be inhibiting the nitrate
reductase activity resulting in the inhibition of growth.

Inorganic phosphate (Chapter III, Section-K) was found to
have a highly promotory effect on growth of tissue. Higher
phosphate levels increased the growth of tissue, phosphate
being one of the most significant mineral nutrient for primary
metabolism in plants.

Calcium, magnesium and iron had adverse effect on growth at
higher levels, because higher concentrations of these elements
adversely affected cell division, chlorophyll formation, etc. in
plants (Chapter III, Section - L).

Further studies on morphogenetic potentiality of tissue
as they age, indicated that the tissue looses its potentiality
to grow fast after several subcultures in the same medium
(Chapter III, Section-M). The growth of tissue started declining
after about 12 subcultures. Similar observations have been made
by White (1967) regarding morphogenetic potentialities of tissue
with age. Murashige and Nakanos (1965) in tobacco; Thomas and
Street (1970) in Atropa and Cheng and Smith (1975) in barley had
also observed that the morphogenetic ability of the tissues
diminishes with the progress of subculture. Further, Torrey (1967);
Lustinec and Horak (1970) and Benerjee and Gupta (1976) have also
.reported that the properties of isolated cells and tissues are
usually lost after variable period in culture. Whether it is
due to changed physiology of the cells or changed chromosome

numbers, or due to other reasons has still remained obscured.

For maintaining the morphogenetic as well as biosynthetic potentiality of the tissue, further work is being carried out to select the stable lines by plating, so that the cell lines can be selected and an actively growing tissue with high capacity for alkaloid production can be maintained.

Section II. (B) Effect of cultural parameters on alkaloid production and tryptophan synthetase activity in Evolvulus alsinoides suspension cultures.

The effect of different parameters on ergot alkaloid production has so far been studied in details in only micro-organisms, especially Claviceps species and Aspergillus species. In present studies the effects of these parameters on the ergot alkaloid production in a higher plant is examined. The results described in Chapter III, in general illustrate a very close similarity in alkaloid biosynthesis in fungi and in Evolvulus alsinoides. Even though the quantity of ergot alkaloids produced in a higher plant is comparatively much less than in Claviceps species, the present investigation clearly indicates that Evolvulus alsinoides, a higher plant belonging to the family Convolvulaceae, shows a similar pattern as in fungi.

"In vivo" studies of Evolvulus alsinoides showed the presence of ergot alkaloids in these plants and this prompted us to carry out further detailed studies in the callus initiated

from young leaves. In comparison, it was also, observed that
the alkaloid production was higher in "in vitro" conditions,
especially in the suspension cultures. Kokate and Radwan (1978)
have also observed that the production of steroidal glyco-
alkaloids was more in callus cultures of Solanum khasianum
than in seeds. The increased synthesis of alkaloids in suspension
cultures might most probably be due to the greater aeration,
easy availability of the nutrients and a larger cell surface
area for absorption. Goldstein et al. (1962) working with
sycamore cambial cells observed less leucoanthocyanins in
static cultures than in cells cultured as suspensions.

Among the auxins studied 2,4-D at 2 mg/l in combination
with 0.4 mg/l kinetin was found to be most effective for enhanced
alkaloid production than any other combinations tested (Chapter
III, Section-C). Working with Datura metel cell suspensions
Chokshi (1975) found that 2,4-D stimulated alkaloid production
more than NAA. Examining the hormonal control of sterioid
synthesis in Solanum xanthocarpum tissue cultures Heble et al.
(1971) further reported that no solasodine was present in tissues
grown on medium containing IAA or IBA. Kinetin also has a
prominent role in synthesis of alkaloids. Steinhart et al. (1964)
have suggested that the control over alkaloid accumulation in
tissues was exerted by the biosynthetic enzymes. Working with
metabolism of hordenine alkaloid in barley roots they demon-
strated that kinetin at lower levels and higher levels controlled
the enzyme activity which resulted in low alkaloid production.

The maximum accumulation of alkaloid was registered on day 25 of culture, thereafter it declined in both static and suspension cultures (Chapter III, Section-C 6). The maximum content of alkaloids were found to occur at the time when cells had completed the most rapid growth phase. Rajbhandari et al. (1969) had observed that alkaloid production was suppressed in actively growing callus and cell suspensions of Atropha belladona. West and Mika (1957) obtained high atropine synthesis in slow growing, differentiated callus of the same species.

Tryptophan synthetase activity was not detected in Evolvulus cultures until day 10 of culture. Further it steadily increased showing maximum activity on day 20 and then declined showing no activity on day 30. Efforts have been made to determine the time course of the appearance and the disappearance of the tryptophan biosynthetic enzymes in Claviceps during the culture period. Several groups have reported an increase in tryptophan synthetase activity at the beginning of idiophase in alkaloid producing strains (Robbers et al., 1972; Schmauder and Gröeger, 1973). As stated earlier, the highest alkaloid production was registered on day 25. The enzyme activity as well as the alkaloid production was more in suspension cultures compared to static cultures (Section C, Results). Examining phenolic compounds in Arachis hypogaea cultures, Shailaja Rao (1979) found that the activities of Phenylalanine ammonia-lyase (PAL) and Tyrosine ammonia-lyase (TAL) attained peak

values on days 10 and 15 respectively, while the maximum accumulation of phenolics occurred on day 20. Thus the high activities of key enzymes involved preceeds the highest accumulation of the product.

Of the sugars tested fructose and glucose, separately and in combination, had adverse effect on alkaloid production in Evolvulus cell suspensions (Chapter III, Section-E 1). Studies on the effect of different levels of sucrose - which proved the best source of carbon and energy, indicated that increasing sucrose levels upto 2% enhanced growth and alkaloid production (Chapter III, Section- E 2). But higher doses of sucrose suppressed the synthesis of alkaloids. Subbaiah (1974) had observed that polyphenol accumulation increased with higher levels of sucrose in Cassia and Datura cultures. Maretzki et al. (1974) also showed that flavanoid synthesis was correlated with carbohydrate constituent in the medium. On the other hand, James (1946) while working with detached leaves of Belladona had observed that sucrose was ineffective for alkaloid production.

Tryptophan synthetase activity in Evolvulus cultures was also found to be regulated by sucrose levels in the medium. No enzyme activity could be detected in absence of sucrose, whereas it was considerable at 1% sucrose level and attained its peak activity at 2% sucrose. Similar observations have been made on PAL and TAL activity in Crotalaria juncea (Shah and

Mehta, 1978 a) and in Arachis hypogaea (Shailaja Rao, 1979).

Nitrogen plays an important role in alkaloid metabolism, as all alkaloids are nitrogenous bases. Nettlship and Slaytor (1974), working with Peganum harmala callus, observed that no alkaloid synthesis occurred in the absence of exogenous nitrogen. In the present studies also it was observed that in the absence of nitrogen there was no alkaloid production (Chapter III, Section-F), whereas increasing concentration of nitrogen upto 840 mg/l induced the synthesis of alkaloids. Shah and Mehta (1978 b) have also reported similar observations on polyphenol accumulation in tissue cultures of Crotalaria juncea.

Attempts were made to examine the effect of non-ionic surface active agents on alkaloid production by facilitating uptake of essential metabolites from the nutrient medium (Chapter III, Section-H). Of the different Tweens tried in the present studies, Tween-80 was more favourable for alkaloid production. Among the different levels of Tween-80 tested, 0.5 v/v was found to be more effective, resulting in a higher yield of alkaloid than control and other Tween levels (Chapter III, Section-H). Experiments in which periodic addition of Tween-80 was made to the cultures showed that highest production of alkaloids occurred when the Tween was incorporated in the medium right from the beginning (Section-H). Chokshi (1975) had noted similar tropane alkaloid enhancement in Datura

callus cultures in presence of Tween-80. Rao and Satyarao (1975) have observed that Tween-80 which also was most effective among the Tweens tested, increased biomass formation and the uptake rate of all components of the medium. Earlier, it was reported by Mizrahi and Miller (1969) that Tweens are not directly involved in the biosynthetic process of ergot alkaloids, but act as surface active agents facilitating the transport of medium components into the cells. Further, Rehacek and Basappa (1971) have demonstrated that Tween-80 was associated with a shift in organic acids and amino acids in cell-pool in Claviceps paspali cultures resulting in increased alkaloid accumulation.

Amino acids are the main precursors of various alkaloids and the influence of different amino acids on alkaloid production is well demonstrated by various workers. The effect of tryptophan incorporation on alkaloid production is examined by some workers (Vining and Nair, 1966; Bu'Lock and Barr, 1968; Taber, 1970; Vining, 1970). In present studies with Evolvulus alsinoides suspension cultures (Chapter III, Section-I), tryptophan treatment resulted in higher production of alkaloids. The growth of tissue was, however, inhibited. Tryptophan enhancement of alkaloid production accompanied by growth supression was earlier demonstrated in certain strains of Claviceps (Floss and Mothes, 1964; Bu'Lock and Barr, 1968).

To elucidate the role of tryptophan further, it was added

periodically to the _Evolvulus_ cell suspensions (Chapter III, Section-I 2). Addition of tryptophan on days 5 (when the cells were in lag phase of growth) and 10 (when cells were embarking upon growth), the alkaloid production enhanced appreciably. On the other hand, later additions of tryptophan on days 15 and 20 (when the cells were in rapid growth phase) had less stimulatory effect on alkaloid production (though it was higher than in the control). As tryptophan suppressed growth, more of it was made available for alkaloid synthesis which, as a result, enhanced. However, rapid growth seemed essential for alkaloid synthesis, for maximum alkaloid production occurred during the period of rapid growth. Similar additions of L-tryptophan to _Claviceps_ cultures, after the end of growth phase and during the alkaloid production phase, had no significant effect on alkaloid production, but a consistent increase was obtained when tryptophan was added at the beginning of the growth phase (Floss and Mothes, 1964).

Stimulation of alkaloid synthesis was also obtained by addition of a tryptophan analogue (5-methyl tryptophan) to _Evolvulus_ cultures, though the analogue was not as effective as the amino acid itself (Chapter III, Section-I 3).

Using radioactive labelling, Floss and Mothes (1964) have, however, observed that 5-methyl tryptophan did not give rise to ergot alkaloid analogues and so its stimulation of alkaloid synthesis could not be explained by a precursor effect. On the

basis of these results, they had proposed that tryptophan had a dual role in ergoline biosynthesis : (i) it is a precursor, (ii) it is in someway involved in the induction of alkaloid synthesising enzymes. Subsequent results from other laboratories have supported the above statement (Bu'Lock and Barr, 1968; Robbers and Floss, 1970; Vining, 1970; Krupinski et al., 1976).

Vining (1970) has argued several causes for the increased yield of alkaloid by tryptophan addition :-

(A) Increased, supply of the limiting substrate,

(B) Depression or activation of the enzyme system,

(C) Overall increase in growth and metabolic activity.

Results obtained in present studies with Evolvulus demonstrated that increased ergot alkaloid production with tryptophan was not due to cause (C) cited above. Enhanced alkaloid production with increasing levels of tryptophan indicated its limiting availability (i.e. A in scheme above).

Studies with Evolvulus alsinoides, further showed that tryptophan synthetase activity declined considerably as the levels of tryptophan increased. Clearly, tryptophan seemed to cause feedback inhibition of its own synthesis. Addition of tryptophan to tobacco, rice, carrot and soybean tissue cultures is known to inhibit anthranilic acid synthesis by feedback mechanism (Widholm, 1971). Similar inhibition of other enzymes

concerned with primary metabolism could explain growth
suppression by tryptophan in present studies (i.e. B in
scheme above).

Addition of mevalonic acid which supplies the "Isoprene"
unit in the formation of ergot alkaloids, resulted in enhance-
ment of alkaloid production (Chapter III, Section-I 4). Though
it was higher than the control, it was much less than in
tryptophan treated cultures. Tryptophan synthetase activity too
was less in comparison to tryptophan treated cultures. The role
of mevalonic acid in ergoline biosynthesis in Claviceps have
been confirmed earlier (Birch et al., 1960; Taylor and Ramstad,
1960, 1961; Baxter et al., 1961).

Next, when the effect of indole, anthranilic acid and
methionine on ergot alkaloid production in suspension cultured
Evolvulus alsinoides was examined, it was found that even
though they are related to alkaloid production, they inhibited
its synthesis. This was probably on account of their inhibition
of tryptophan synthetase activity as also growth (Chapter III,
Section-I 5-9). Working with Aspergillus fumigatus Rao and
Patel (1974) also observed that the above precursors inhibited
alkaloid production.

Studies on amino acids (Chapter III, Section-J) showed
that tryptophan, methionine and serine were present in maximum
quantities in cells at different periods. The presence of
tryptophan at its maximum concentration on day 20 of culture,

clearly corresponded with the peak value of tryptophan
synthetase activity on day 20 and maximum alkaloid production
on day 25. Further, the presence of methionine and serine in
maximum quantities on day 15 of culture also suggested their
significant role in alkaloid production - serine being
converted to tryptophan and methionine being used for
supplying the methyl group of ergot alkaloids.

As stated in the Introduction, the production of secondary
metabolic products in microorganisms is commonly associated with
slow growth and with low concentration of orthophosphate in the
medium. Studies carried out with Evolvulus alsinoides suspension
cultures to find out the effect of high concentration of
inorganic phosphate revealed that at the highest level of
phosphate tested, alkaloid production was drastically reduced
and was much below the control (Chapter III, Section-K 1).
Similar phenomenon was found in Claviceps and other micro-
organisms (DE Waart and Taber, 1960; Robers et al., 1972).
With increasing phosphate levels, the growth of suspension
cultured Evolvulus was, however, considerably enhanced.

This inhibitory effect of high inorganic phosphate on
alkaloid production was found to be restored by addition of
L-tryptophan (Chapter III, Section-K 2). On the contrary,
mevalonic acid or 5-methyl tryptophan had no counter-effect
on phosphate inhibition of alkaloids (Chapter III, Section-K
3, 4). These observations suggested that the high phosphate

inhibition of alkaloid production was mediated in someway
through tryptophan. Weygand and Floss (1963) have mentioned
the following aspects of phosphate inhibition of alkaloid
synthesis in Claviceps :

(i) Higher phosphate levels could directly block the
induction of the alkaloid synthesising enzymes or one of the
steps in the biosynthetic pathway,

(ii) High phosphate could influence alkaloid production by
causing a depletion of endogenous tryptophan pool or preventing
tryptophan accumulation.

Our present studies with Evolvulus alsinoides suspension
cultures showed clearly that as the levels of inorganic
phosphate was increased in the medium, the tryptophan synthetase
activity started declining recording no enzyme activity at the
highest level tested. This suggested that higher levels of
inorganic phosphate inhibited tryptophan synthesis and hence
the alkaloid production was limited. The results obtained in
the present studies therefore, lend greater support to the
second possibility.

The study of tryptophan synthetase activity in cultures
containing higher inorganic phosphate along with mevalonic acid
or 5-methyl tryptophan also showed inhibition of the enzyme
activity. The above additives could not induce the alkaloid
production inhibited by higher levels of inorganic phosphate.
This further supported the above view that higher levels of

inorganic phosphate inhibited tryptophan synthetase activity
and as a result tryptophan synthesis was blocked which caused
the inhibition of alkaloids.

As mentioned earlier 5-methyl tryptophan induced alkaloid
production in standard medium (Chapter III, Section-I 3).
However, it could not restore the inhibition of alkaloid
production caused by high phosphate level, which tryptophan
could do. This seemed to imply that 5-methyl tryptophan can not
substitute for tryptophan. It might probably be exerting an
inducing effect in cultures with normal levels of phosphate,
where tryptophan was not limiting. On the other hand, in high
phosphate levels, 5-methyl tryptophan could not induce alkaloid
production as tryptophan was not available.

In absence of studies with the effects of increasing
phosphate concentration on enzymes inducing ergot alkaloid
synthesis, the first possibility can not be considered with any
degree of confidence.

There could also be other possibilities; for Demain (1972)
has proposed that phosphate inhibition might involve regulation
of energy charge. The relative levels of ATP,ADP levels in the
cells would involve activation and inhibition of enzymes of
primary metabolism.

The first specific enzyme in the ergoline pathway has been
identified to be Dimethylallyl pyrophosphate : L-tryptophan
dimethylallyl transferase (DMAT synthetase). Lee et al. (1976)

have detected the presence of DMAT synthetase in cell free extracts of $C_{laviceps}$ sp; strain SD.58, catalysing the formation of DMAT from dimethylallyl pyrophosphate and L-tryptophan. This enzyme was found to be activated by Fe^{2+}, Mg^{2+} and Ca^{2+}. We could not study the activity of this enzyme directly due to unavailability of tracer technique facilities, but the influence of Fe^{2+}, Mg^{2+} and Ca^{2+} was examined (Chapter III, Section-L). It was observed that alkaloid production enhanced with increasing Ca^{2+} levels. At lower levels, Mg^{2+} stimulated alkaloid production, but at its higher levels the alkaloid production was reduced. Same pattern was noticed also with Fe^{2+}. Similar observations have been made with Claviceps species by Lee et al. (1976). This seemed to indicate, though indirectly, that DMAT synthetase was regulated in Evolvulus alsinoides suspension cultures also by the availability cofactors like Ca, Mg and Fe, of which Ca in particular seemed to be more limiting.

Taking into cognisance the overall results presented in this thesis, the following broad conclusions can be derived :-

(i) Like ergot fungus, few higher plants can also synthesise ergot alkaloids. The chemotaxonomic significance of limited distribution of this biosynthetic capability in a few members of the family Convolvulaceae, however, remains still obscure.

(ii) There is a close similarity in the manner in which

the different cultural parameters influence the alkaloid
production in fungi as well as higher plant. These include
mainly the relationship between growth phases and alkaloid
synthesis, pattern of tryptophan synthetase activity, phosphate
inhibition of alkaloid biogenesis and its restoration by
tryptophan and its analogues.

(iii) Plausible explanation of phosphate inhibition of
alkaloid production is presented; i.e. it causes suppression
of tryptophan synthetase activity which results in non-
availability of tryptophan, the key precursor of ergot alkaloids.

(iv) Methyl-tryptophan cannot substitute for tryptophan
in overcoming high phosphate inhibition. However, it seemed to
induce alkaloid synthesising enzymes in presence of normal
dose of phosphate.

(v) Studies with cofactors of DMAT-synthetase offer
indirect evidence of its regulatory role in higher plants also.

(vi) Role of hormonal and nutritional (carbohydrates,
nitrates, macro- and micro-element salts) status of the medium
in enhancing alkaloid production is also envisaged in the
present studies.

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