SYNTHETIC BRASSINOSTEROIDS : ITS ROLE IN A FEW PLANT GROWTH PROCESSES

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SYNTHETIC BRASSINOSTEROIDS : ITS ROLE IN A FEW PLANT GROWTH PROCESSES

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Affectionately

Dedicated to My Beloved

Parents

&

Brothers

DEPARTMENT OF CROP PHYSIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **"SYNTHETIC BRASSINOSTEROIDS: ITS ROLE IN A FEW PLANT GROWTH PROCESSES"** submitted by **Mrs. ANITHA NAREN** in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in CROP PHYSIOLOGY to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by her under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or other similar titles.

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ABBREVIATIONS AND SYMBOLS

ABA	-	Abscisic acid
ABPs	-	Auxin binding proteins
ADP	-	Adenosine diphosphate
ALP	-	Alkaline phosphate
AMP	-	Adenosine monophosphate
ATP	-	Adenosine triphosphate
BA	-	Benzyl adenine
BCIP	-	5-Bromo-chloro-3-indolyl phosphate
BR	-	Brassinolide
BRs	-	Brassinosteroids
BSA	-	Bovine serum albumin
C	-	Degree centigrade
CH	-	Cycloheximide
Cm	-	Centimeter
$CoCl_2$	-	Cobalt chloride
Conc	-	Concentration
CPM	-	Counts per minute
CPZ	-	Chlorpromazine
DAS	-	Days after sowing
2,4-D	-	2,4-dichlorophenoxyacetic acid
o	-	Degree
DCCD	-	Dicyclohexylcarbodiimide
DMF	-	Dimethylformamide
EDTA	-	Ethylene diamine tetraacetic acid
EGTA	-	Ethylene glycol-bis(aminoethyl) tetraacetic acid
ELISA	-	Enzym linked imminosorbent assay
FCA	-	Freunds complete adjuvant
Fig	-	Figure
5-FLU	-	5-Fluorouracil
g	-	gram
GA	-	Gibberellic acid
Gal	-	Galactose
HBR	-	homobrassinolide
hrs	-	hours
IAA	-	Indole-acetic-acid
IgG	-	Gamma-imminoglobin
KCl	-	Potassium chloride
KCN	-	Potassium cyanide
$LaCl_3$	-	Lanthanum chloride
m	-	moles
M	-	Molarity
mg	-	milligram
µg	-	microgram
min	-	minutes
ml	-	milliliter
µl	-	microliter
mm	-	millimeter
mM	-	millimolar
µM	-	micromolar
µ	-	micron

mRNA	-	messanger RNA
NAA	-	Naphthalene acetic acid
NET	-	Nitro blue tetrasolium
ng	-	nanogram
nM	-	nanomole
OD	-	Opticl density
PBS	-	phosphate buffered saline
PCIB	-	p-chlorophenoxyisobutric acid
%	-	per cent
PGM	-	Pollen growth media
pH	-	potential of hydrogen
POPOP	-	1,4 bis[2-(5-phenyloxazolyl)]-benzene phenyl oxazolylphenyl-oxazolyl-phenyl
pM	-	picomole
PNPP	-	Paranitrophenol phosphate
PPO	-	2,5 Diphenyloxazole
ppm	-	parts per million
RBD	-	Randomized block design
RH	-	Relative humidity
RIA	-	Radioimmino assay
rpm	-	rounds per minute
RNA	-	ribo nucleic acid
RR	-	Ruthenium red
SDS	-	Sodium dodecyl sulphate
STS	-	Silver thiosulphate
2,4,5-T	-	Trichlorophenoxyacetic acid
TPZ	-	Trifluoperazine
Trp	-	Tryptophan
TSS	-	Total soluble solids
ZB	-	Zero binding

INTRODUCTION

I INTRODUCTION

Brassincomplex - a novel plant growth promoting steroidal lactone, was first isolated and identified from pollen grains of Brassica plants (Brassica napus) by Mitchell et al., (1970). A number of related compounds were then identified from a wide variety of plant species and they were collectively called as brassinosteroids (BRs). Till now fifteen BRs have been characterized from fourteen plant species and are present in all parts of the plant at very low levels.

BRs promote a dual response of cell division and cell elongation resulting in both enhancement and acceleration of the overall growth of the plant.

There is a need for a more versatile test system for the discovery of brassins and the subsequent isolation and characterization of the active component that would permit the study and better understanding of their interactions with other hormones and related growth substances.

This group of compounds showed growth regulatory effects in plants, similar to that of endogenous plant hormones, auxins and gibberellins. A number of scientists working at different parts of the world, showed that BRs act synergistically with auxins and gibberellins in inducing cell elongation.

In 1972, the US Department of Agriculture reported that the biological effects of crude brassinolide extract of rape pollen (Brassica napus) on growth of young bean plants and Siberian elm trees, and showed that this chemical caused overall increase in growth. In many greenhouse trials, synthetic BRs analogues were shown to boost the yield significantly of various vegetables and root crops, like radish, lettuce, pepper and potato. Studies conducted in Japan by several research groups identified that seed treatment with BR results in growth acceleration of rice and tobacco. This effect was prominent under low temperature conditions. Seedling treatment was found to be effective in increasing the yield of potato and sweet potato. This group of chemicals are also shown to protect plants from environmental stresses like low temperature, herbicides, salt and pathogenic micro organisms. It is necessary to know the exact mode of action and its biological activity and also the agricultural application of this new class of growth substance. Keeping this in view, the present investigation was carried out with the following objectives.

1. To study the influence of BR on growth responses using a few test systems.
2. To study the interaction of BR with auxins and other hormones on growth responses using a few test systems.
3. Mode of action of BR in induction of growth responses.
4. To study the agricultural application of BRs: Influence of BR on growth and productivity in sunflower and grapes.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE

Brassinosteroids are endogenously occurring growth regulating substances in plants. Upto 1980s, lot of interest was shown in understanding the biological activity and the agricultural uses of brassinosteroids. Over the last few years, there has been increased interest on the physiological role of brassinosteroids, their interaction with other plant hormones and also in understanding the mode of action of these groups of compounds in inducing growth responses.

In this review, an attempt is made to give a brief account of the literature available on various aspects of brassinosteroids. The objective of this review is to touch on as many areas as possible, which gives some background information on the various aspects of research undertaken in this study to know the biological activity and mode of action.

CHEMICAL STRUCTURE AND PROPERTIES OF BRASSINOSTEROIDS

CHEMICAL STRUCTURE AND TYPES

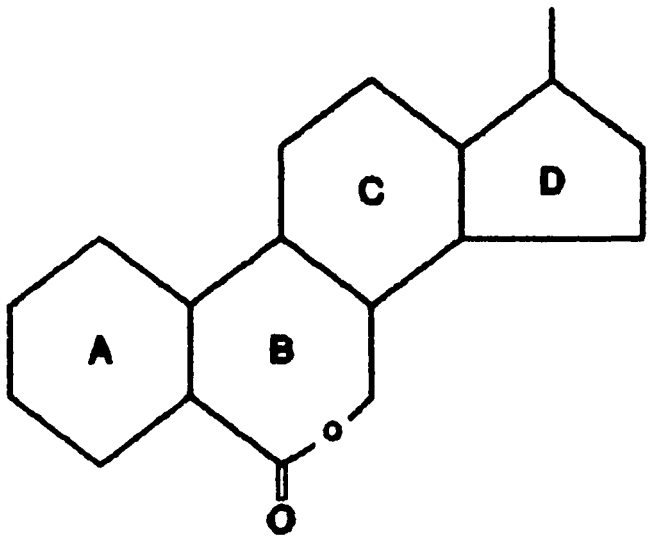
Brassinosteroids (BRs) are a new class of naturally occurring polyhydroxy steroids. Till now, sixteen naturally occurring BRs are identified, out of which fifteen have been identified from higher plants and one

from a lower plant and they are designated as BR_1, BR_2, ,..... BR_n (Yokota and Takahashi, 1986). In 1988, Yokota et al. reported the occurrence of over thirty different types of BRs. All the identified and characterized BRs from plants are 5α -cholestane derivatives. Based on chemical structure, number of carbon atoms, and structural differences, these BRs were classified into three different categories.

Based on chemical structure, the BRs were classified into lactones, ketones and 6-deoxo compounds (Adam and Marquardt, 1985). Activity decreases from lactones to ketones to 6-deoxo compounds. BRs containing a steroidal nucleus with lactone, exhibits greater activity compared to ketones. The 6-deoxo compounds possess least activity due to lack of functional oxygen group in the lactone or ketone form. The basic structure of BRs i.e., lactone, ketone and 6-deoxo compounds are shown in Fig. 1.

Based on the number of carbon atoms, BRs are classified into three groups (Yokota and Takahashi, 1986) C_{27}, C_{28} and C_{29} steroids, similar to the classification of phytosterols. The variations in number of carbon atoms occur primarily because of different substitutions at C-24 position. If there is no substitution at C-24 position that becomes C_{27} steroid. Substitution of methyl or an exomethylene group leads to C_{28} steroids. Substitution of ethyl or an ethylidene leads to C_{29}

a.



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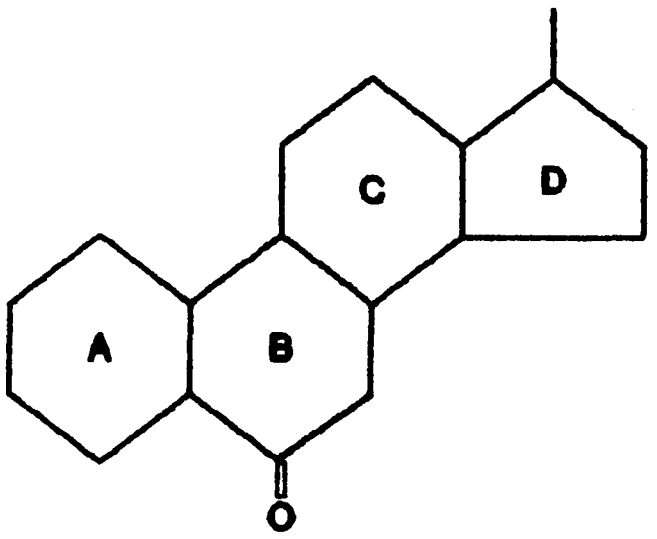

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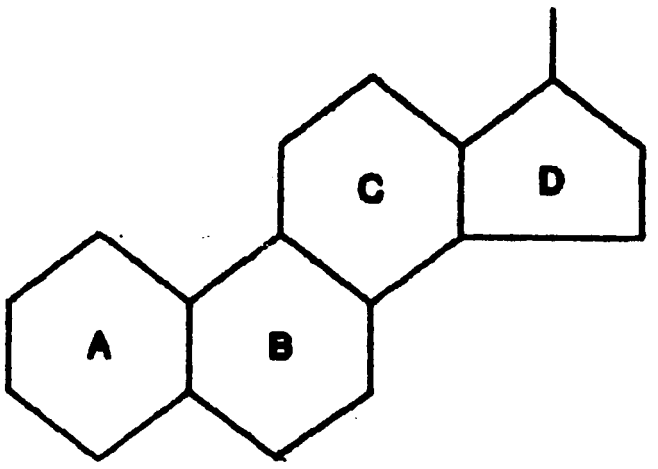


Fig. 1: Basic structure of Brassinosteroids a)lactone, b) ketone, and c) 6-deoxo compound.

steroids. All BRs carry a vicinal glycol in the side chain at C-22R and C-23R. Thirteen other BRs have another vicinal glycol group at C-2α and C-3α in A-ring. The two other BRs, typhasterol and teasterone carry a 3α - hydroxyl and 3β -hydroxyl group, respectively. This leads to the speculation that the introduction of a vicinal glycol occurs first in the side chain and then in the A-ring. Typhasterol and teasterone might be considered as precursors of BRs (Abe et al., 1984a; Schneider et al., 1983; Yokota et al., 1983a). Based on structural differences and activity, these BRs are classified into three groups - Brassinolide, Homobrassinolide and Epibrassinolide (Adam and Marquardt, 1985). They differ in structure only at C-24 position. Physiological activity reduces from brassinolide to homobrassinolide to epibrassinolide. Homo and epi brassinolide are synthetic ones. Brassinolide occurs in nature and can also be synthesized (Fig. 2). However, in some bioassays all three were found to have similar activities.

PHYSICAL AND CHEMICAL PROPERTIES OF BRASSINOLIDE

Formula	$C_{28}H_{48}O_6$
Melting point	274 to 275 °C
Solubility	Methanol or Ethanol
Translocation	Readily translocated
Stability	Stable in acidic condition
Concentration present in pollen	Very very low (200 ppb to 10 ppm)
Absorption peak	207 nm (uv range)
Effective concentration range	0.005 to 0.1 µg

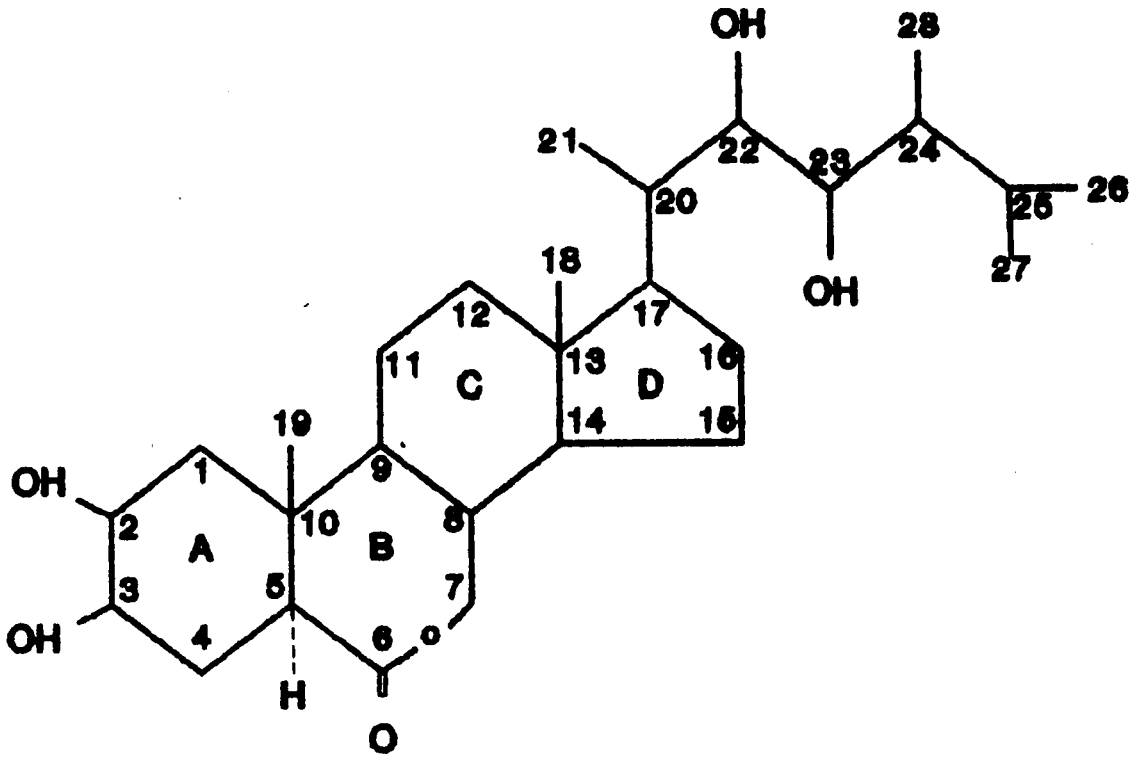


Fig. 2 : Basic structure of Brassinolide.

STRUCTURE - ACTIVITY CORRELATIONS

A number of BR analogues have been synthesized and examined for their structure-activity correlations by means of the bean second internode assay and bean first internode assay (Thompson et al., 1982) and rice leaf lamina inclination assay (Kondo and Mori, 1983; Takatsuto et al., 1983a; Wada and Marumo, 1981; Wada et al., 1983). In these bioassays the following structural features were indicated as necessary for the activity:

1. A trans A/B ring system (H atom at C_5, A/B ring junction),
2. A 7-oxalactone or a 6-ketone system in the ring B,
3. Cis α -oriented hydroxyl groups at C-2 and C-3,
4. Cis hydroxyl groups at C-22 and C-23, and
5. A methyl or an ethyl substitution at C-24.

SYNTHETIC BRASSINOSTEROIDS

Many research groups in different laboratories examined the biological activity of naturally occurring BRs, synthesized their analogues and tested their activity in biological systems (Mitchell et al., 1970; Worley and Mitchell, 1971; Mori, 1980). One of the most important aspects in synthetic BR is the conversion of 3β-hydroxy groups to 3α-hydroxy group. Wada and Marumo (1981) focussed their attention to the synthesis of brassinolide analogues with modified A-ring and studied

their biological activity. One among those synthesized by Wada and Marumo (1981) showed one tenth of the activity of Brassinolide in leaf lamina inclination test on rice seedlings. A number of other analogues with modified A-ring, were poorly active, and an analogue with modified B-ring showed only one hundredth of the activity of its 1-oxo-ketone isomer. This suggests that the 2α, 3α - dihydroxy-7-oxo-6-ketone moiety is one of the requisites for the biological activity.

Takatsuto et al. (1984) is an another active research group involved in the synthesis of BR analogues at Tokyo Institute of Technology. They have synthesized a number of new synthetic BR analogues and showed that some of these analogues exhibited almost similar activity as that of brassinolide by both Raphanus and rice leaf lamina inclination tests.

The chemical name of a few of the synthetic BRs are given below :

(22R, 23R, 24S)-6-β-Methoxy-24-methyl-3α,5-cyclo-5α - cholestan- 22, 23-diol,

(22R, 23R) -24-Epibrassinolide,

(22S, 23S)-24-Epibrassinolide,

26, 27-Bisnorbrassinolide, and

(22S, 23S)-2-deoxy-24-Epibrassinolides.

OCCURRENCE AND DISTRIBUTION OF BRs IN PLANTS

Immediately after the pioneering work of Mitchell et al. (1970), in the identification of steroidal lactone from pollen extract, the occurrence of similar substances in micro organisms and higher plants, were reported by many workers. A number of steroids related to BR have been isolated from various plant sources. By 1985 fifteen BRs have been characterized from fourteen plant species including eleven angiosperms (eight dicots, three monocots) (Abe et al., 1984a; Grove et al., 1979; Schneider et al., 1983), two gymnosperms (Yokota et al., 1983a; Yokota and Takahashi, 1986) and one alga (Yokota et al.,1985,un publ). These findings coupled with bioassay data of Mandava and Mitchell(1971), suggest that BRs may occur more widely amongst plant species.

BRs are shown to be present in all parts of the plants. Higher levels of BRs were found in various pollen, indicated that pollen is one of the richest source of BRs. This observation made Sasse (1985) to see the effect of BR on pollen tube growth. He also found that pollen tube growth was promoted by nM levels of synthetic 24-epimere of brassinolide, suggesting that BR might be concerned with physiology of reproductive growth. Horgen et al. (1983) showed the presence of higher level of BRs in roots also. Immature seeds also contain high levels of BRs. Yokota et al. (1984)

identified eight BRs from seeds of Dolichos lablab. Seeds of Phaseolus vulgaris also exhibit high BR activity. The concentration of BRs in vegetative tissues are rather low compared to the concentration in pollen or in immature seeds. Arima et al. (1984) and Grove et al. (1979) showed that insect galls also contains higher amounts of BRs than normal tissue. Similarly, crown gall cells were also shown to contain higher amounts of BRs (Park et al., 1989). This suggests the possibility that BRs might be concerned with abnormal growth of plant tissues.

BIOLOGICAL ACTIVITY OF BRASSINOSTEROIDS

The discovery of a lipoidal complex, possessing powerful growth promoting properties from the pollen of rape (Brassica napus.L) by Mitchell et al. (1970) led to an intensive effort to characterize its range of effects. The extract termed "brassins" or "brassin-complex" was shown to elicit both cell enlargement and cell division in both longitudinal and lateral planes (Mitchell and Gregory, 1972; Worley and Mitchell, 1971 and Worley and Krizek, 1972). This cell elongation and cell division leads to the unusual growth effects like swelling, curvature and splitting of second internode in bean (Grove et al., 1979) and Worley and Mitchell (1971) showed marked changes in the vascular anatomy of bean leaves.

BR has got an independent effect on rice leaf lamina inclination. Wada et al. 1981 showed that BR and its synthetic derivatives have strong activity in rice leaf lamina inclination test and this test is simple and specific for BR, and was used as a micro quantitative estimation of BRs (Wada et al., 1984). This test was also used as a method for detecting antibrassinolide compounds. Cerana et al. (1983) showed that BR induces a significant stimulation of root growth associated with an increase in acid secretion, but auxin inhibits such action. However, Cerana et al. (1983b) showed both IAA and BR are effective in inducing growth and acid extrusion in azuki bean epicotyls. It suggests that in roots, the action of BR and auxins are elicited through different pathways. Yokota (1985) showed BR was not effective in avena curvature assay when tested alone or in combination with IAA.

BR activity was compared with auxin in a number of bioassay systems. Yopp et al. (1981) showed that differences exist between the effects of auxin and BR in selected auxin bioassays like retardation of dwarf bean hypocotyl hook opening, elongation of maize mesocotyl, pea epicotyl, azuki bean epicotyl sections, fresh weight increase in Jerusalem artichoke and pea epicotyl sections. The azuki bean and dwarf pea epicotyl bioassays were much more responsive to BR and IAA. Shen et al.

(1988) showed that epi-BR enhanced the elongation of wheat coleoptile segments and was more effective between 10^{-6} to 5 ppm. The lag phase of IAA promoted elongation was shorter than that of epi-BR promoted elongation.

Arteca et al. (1983) found an increase in production of ethylene by etiolated mung bean tissue as a result of treatment with BR. But Eun et al. (1989) failed to show a similar effect. They showed that BR mediated the regulation by altering the levels of endogenous IAA and ABA. BR treated segments showed higher level of IAA and elevated levels of ABA than water treated segments. Xu and Zaho (1989) showed that peroxidase and IAA oxidase activities were greatly reduced by 0.1 ppm BR treatment of the hypocotyl of cucumber seedlings and peroxidase activity completely ceased at greater than 1.0 ppm concentration of BR.

BIOLOGICAL INTERACTION EFFECT OF BRASSINOSTEROIDS WITH OTHER HORMONES

A powerful synergism between BR and IAA was observed in many systems like azuki bean, pea epicotyl and bean hypocotyl hook bioassays (Steffen et al., 1979; Yopp et al., 1981 and Chio et al., 1990). Iwata and Stowe (1973) showed synergism in oleanimins also.

BR was compared with gibberellins and cytokinins in a number of bioassay systems. Elongation of excised,

etiolated bean hypocotyl and dwarf pea epicotyl in the light, was shown to be enhanced by gibberellic acid(GA) and brassin complex (Yopp et al., 1979a; Mandava et al., 1981). BR as well as gibberellic acid were reported to induce cell elongation in epicotyls of mung bean plants (Gregory et al., 1979). In a few systems gibberellic acid resembles BR. Like BR, GA also inhibits betacyanin accumulation in amaranths seedlings and prevented adventitious root inhibition in hypocotyls of mung bean, dwarf bean and cucumber (Mandava, 1988). BR does not interact synergistically with gibberellin GA_3. However, in biological system additive effect of gibbberellins and BR has been shown.

BR shows cytokinin like activity in cucumber cotyledon expansion assay. Wada et al. (1985) found that BR is quite active in wheat leaf unrolling test, in which cytokinin bioassays involving expansion of dwarf pea epicotyl hooks, dark synthesis of betacyanin in amaranths and retardation of xanthium leaf disc senescence (Mandava et al., 1981).

MODE OF ACTION OF BR AND IAA IN CELL ELONGATION

BR has growth promoting effects similar to auxins and gibberellins. Yopp et al. (1981) showed similarity between the effects of auxin and BR in selected auxin bioassays. Cerana et al. (1983a) reported that IAA and BR

act in an additive manner in azuki epicotyl section elongation, and concluded that the action of BR is independent from that of IAA. However, they did not test the interaction by changing the IAA concentration. Romani et al. (1983) also reported that BR and auxin act independently on the basis of the findings obtained in maize root segments. BR stimulated elongation was inhibited by IAA. But, Yopp et al. (1981) and Katsumi (1985) pointed out the strong synergism that exists between auxin and BR. This synergism, however, occurs only when tissues are treated first with BR and then with IAA, BR is ineffective when the order of treatment is reversed (Cohen and Meudt 1983; Katsumi 1985). This suggest that BR had no effect in elongation induced by IAA pretreatment, and also indicates that cells which had already started to elongate were not the target cells of BR. More important is that BR elicited growth responses, such as hypocotyl elongation and rice leaf lamina bending, are nullified by the antiauxins, p-chlorophenoxy-isobutyricacid (PCIB) or 2,3,5 - triiodo benzoicacid (TIBA) (Katsumi, 1985; Takeno and Pharis, 1982; Yopp et al., 1979a). This suggest that BR enhances the sensitivity of plant tissues to auxin, resulting in synergistic interaction with endogenous auxin, but does not itself have auxin like function.

The synergistic interaction of BR was also observed with synthetic auxin, 2,4-D. Arteca et al. (1983), Yopp et al. (1981), Cohen and Meudt (1983) showed synergism with synthetic auxins. This indicated that the action of BR is not related to the metabolism of IAA. In fact, Cohen and Meudt (1983) demonstrated that BR neither affects IAA synthesis nor its break down in the first internode section of Phaseolus vulgaris.

BR showed growth promoting effects similar to gibberellins also. BR elicits pronounced stem elongation of dwarf pea hypocotyls, dwarf bean apical segments (Mandava et al., 1981) and mung bean epicotyls (Gregory and Mandava, 1982) which are sensitive to GAs, but not to auxins. These authors and Katsumi (1985), reported that the effects of BR and GA are additive. HBR and GA have similar pretreatment effects. They appear to have an independent mechanism of action. The HBR effect can be reduced by washing the sections with water but not that of GA, suggesting that the effect is reversible and the binding of HBR molecules to the action site might be loose. A membrane bound ATPase inhibitor, dicyclocarbodiimide inhibits BR elicited growth responses in cucumber hypocotyl sections but not GA_3 induced growth. Ancymidol, a growth retardant, inhibits GA-mediated growth response, but had no influence on the effect of BR (Gregory and Mandava, 1982; Grove et al., 1979). Takeno and Pharis

(1982) have reported that BR is almost ineffective in the dwarf rice seedling elongation test, which is known to be quite sensitive to GA. These observations indicate that the mode of actions of BR and GA are independent.

Earlier work also indicated the need for protein synthesis in BR induced effects (Cerana et al., 1983; Kalinich et al., 1985, 1986). Kulaeva et al. (1989) showed that treatment of wheat leaves with BR led to altered gene expression. Clouse et al. (1990) proved that BR induces protein synthesis both at transcription as well as translation levels. They reported the in vitro synthesis of new polypeptide from RNA . induced by BR treatment in soybean tissue as well as same clone regulation of a few other polypeptides.

These informations indicate that BR might be inducing the response by acting at genome level. Such possibilities were postulated by Sasse (1991). The sequence of events leading to gene expression and protein synthesis can be postulated for BR induced synthesis of new transcripts.

MODE OF ACTION OF AUXINS

Auxin induced cell elongation has been studied extensively since many years in different young elongating plant tissues. The tissues often used to evaluate auxin induced cell elongation are coleoptiles,

hypocotyl and epicotyl tissue segments. The mode of action of auxins in inducing cell elongation in these test systems are reviewed extensively by Brummel and Hall(1987), Guilfoyle (1986) and Hathway (1990). It is generally agreed that the response of many types of stem tissue cells to auxins is biphasic. The initial rapid phase of the response is similar to growth that can be induced by low pH, and the second delayed growth phase has properties similar to other long term auxin responses such as cell division, differentiation and morphogenesis (Evans, 1980).

The molecular mechanism by which auxins causes cell extension is unknown. In auxin induced rapid responses leading to cell elongation, it was proposed that auxin either directly activate a membrane bound H^+ pump or cause H^+ extrusion by a mechanism requiring synthesis of specific proteins or regulate the amount of proteins affecting cell wall rigidity (Moore, 1989; Theologis, 1986). Using two dimensional gel electrophoresis analysis of *in vitro* translation products encoded by mRNA extracted 30 to 60 minutes after auxin treatment showed increased amount of several transcripts (Theologis, 1986; Zurfluh and Guilfoyle 1982a). Some of the genes encoding for these transcripts have been cloned and these genes were shown to be subject to rapid (5 to 10 minutes) auxin dependent transcriptional regulation (Conner *et al.*, 1990; Hagen *et al.*, 1984; McClure and Guilfoyle

1987; McClure et al., 1989). However, these specific functions in the first phase of auxin stimulated cell elongation yet to be shown. In contrast findings of Dietz et al. (1990) and McClure and Guilfoyle (1989) show that early mRNA increase was not specific for regions undergoing cell extension.

For auxin induced increase and sustained elongation growth for a long period, the requirement of continuous protein and RNA biosynthesis has been shown (Key et al., 1967; Moore 1989). The mechanism involved in auxin induced mRNA accumulation could be transcriptional activation of auxin regulated genes (Hagen and Guilfoyle 1985; McClure et al., 1989), post transcriptional processing of mRNAs (Hagen et al., 1984; Hagen and Guilfoyle 1985) and transport of mRNAs from the nucleus to the cytoplasm or alterations in the life time of mRNAs.

In a few test systems auxin regulated genes have been identified and the cDNA and gene sequences have been reported (Ainley et al., 1988; Czarnecka et al., 1988; Hagen et al., 1988; McClure et al., 1989). However, exact function or role of these auxin regulated gene products still remains a mystery. It is also not known which cellular protein functions in auxin induced cell elongation (Guilfoyle et al., 1990). Similarly, very little is known about the signal transduction pathway

involved in auxin action. Many scientists now believe that at least some of the auxin-regulated genes which have now been positively identified, prove to play some role in auxin induced elongation growth. Identification of the cis- and trans- acting elements that cofactor auxin inducibility to auxin regulatory genes, should provide some insight into one or more of the signal transduction pathways involved in auxin regulated gene expression.

IDENTIFICATION AND CHARACTERIZATION OF AUXIN-BINDING SITES

The plant hormone auxin (Indole-3-acetic acid) in higher plants regulates normal growth and development by cell elongation, division and differentiation (Guilfoyle, 1986). Rapid responses to auxin have been observed in organelles such as plasma membrane, golgi apparatus, endoplasmic reticulum (ER) and nucleus as well as in the cell wall. The specificity of auxin is supposed to be due to the presence of specific receptors (Rubery, 1981). However, it is unknown whether the diverse responses of different organelles to auxin are mediated by a single or multiple species of receptors. Specific binding sites for auxin have been demonstrated in both soluble and membrane fractions of various plant tissues (Hertel et al., 1972; Ray et al., 1977b). Batt et al. (1976) showed the existence of two classes of high affinity auxin binding sites in crude membrane preparation from corn coleoptiles.

Batt and Venis (1976) showed evidence from enzymic and chemical assays and from electron microscopy suggests that site 2, the auxin specific binding site is located in fractions enriched in plasma membrane, whereas site 1 is associated with golgi membranes and/or endoplasmic reticulum. Finally, Dohrmann et al. (1978) reported the presence of three types of auxin binding sites in different membrane fractions of maize coleoptiles i.e., site 1 in the endoplasmic reticulum, site 2 in the tonoplast and site 3 in the plasma membrane. Site 3 has been shown to be the auxin-transport system in closed membrane vesicles (Hertel et al., 1983).

AUXIN-BINDING SITES IN OTHER TISSUES

Ray et al. (1977b) showed high affinity of naphthalene acetic acid (NAA) binding in microsome preparation from coleoptiles, primary leaves, and mesocotyls. Moloney and Pilet (1981), showed high affinity binding of auxin seems to be associated with a 10,000 g rather than with a 48,000 g pellet, suggests that the binding sites are not associated with the endoplasmic reticulum, but presumably with the plasma membrane. They found IAA has higher affinity to this site than that of NAA and much higher than that of 2,4-dichorophenoxy acetic acid (2,4-D) from displacement experiments.

Battacharyya and Biswas (1978) have demonstrated high affinity IAA binding to crude membrane fractions of oat roots and also showed auxin-binding to oat root membranes specific for IAA only. Hertel et al. (1972) detected binding of labelled NAA to particulate material in homogenates of maize coleoptiles. This binding is due to receptor sites for plant hormones of the auxin class. Ray and Dohrmann (1977) have detected properties of the auxin binding sites that occur in maize cellular membranes. Binding of IAA to microsome preparations from cotyledonary buds of pea was shown by Jablonovic and Nooden (1974). After incubation of membranes with ^{14}C labelled IAA, only 0.5 - 1.7 per cent of the total amount of bound radioactivity could be replaced by excess of IAA or NAA. After washing of the incubated membranes, most (85 %) of the labelled IAA disappeared, suggesting binding was compatible and most of the non-specific binding was removed by washing while high affinity binding remained.

In 1985, Lobler and Klambt purified a 20 kDa subunit ABP from a maize membrane fraction and purified by affinity chromatography to separate two pools of proteins, containing binding or non-binding activities. For further purification polyclonal antibodies were raised against each of these crude fractions and used in immuno affinity columns. The purified sample bound NAA as a single class and with the same affinity, pH optimum and

specificity as site 1 activity in crude extracts. It had a native molecular mass of 40 kDa. Shimomura et al. (1986) purified ABPs in maize by ion exchange and affinity chromatography, this ABP has a native molecular mass of 42 kDa and the aminoacid composition of this ABP was also determined.

THE PHYSIOLOGICAL ROLE OF CALCIUM AND CALMODULIN IN PLANTS

The regulation of biochemical processes by calcium and calmodulin is of great biological significance in the growth and development of plants and animals (Cheung, 1980; Hanson, 1983). In plants, growth and development in response to hormones and external stimuli such as gravity and light are found to require the mediation by calcium and calmodulin (Leopold, 1977; Poovaiah and Leopold, 1973). Calcium has been suggested to act as a secondary messenger in plants (Hanson, 1983). Calcium ATPase (Fukumoto and Nagai, 1982), NAD-Kinase (Anderson and Cormier, 1978), Protein kinases (Hetherington and Trewavas, 1982; Veluthambi and Poovaiah, 1984), isofloridoside-phosphate synthase (Kauss, 1983) and quinate NAD + oxidoreductase (Refeno et al., 1982) are some of the enzymes reported to be promoted by calcium and calmodulin in plant systems.

INHIBITORS OF CALCIUM AND CALMODULIN

Many physiological and biochemical processes in plants are mediated by calcium and calmodulin protein. The general mechanism and the mode of action of calmodulin in regulating physiological and biochemical processes are studied by using a number of compounds. These substances in general can be called as calcium inhibitors, which might block calmodulin activity in many ways (Poovaiah, 1988).

The role of calcium in cellular process can be studied by the use of several inhibitors with specific mode of action. Several natural and synthetic inhibitors are now available. They are:

1. Calcium chelators - Prevent the availability of calcium pools by specifically chelating their charges, Eg: EGTA,
2. Calcium channel blockers - By preventing the entry of the ion through specifically blocking the ion channels. Eg: verapamil, RR and lanthanum chloride, and
3. Calcium calmodulin inhibitors - Inactivating the calcium calmodulin protein by binding to the calmodulin protein. Eg: chlorpromazine (CPZ).

1. Calcium chelators

Ethylene glycol-bis(aminoethyl)tetraacetic acid (EGTA) is a specific chelator of calcium ions (Reed and Bygrave, 1974b). The two carboxylic groups of EGTA forms electrostatic bonds with calcium whilst, two coordinate

bonds are formed between calcium and the two nitrogen atoms. This results in the formation of very stable compound which is highly soluble in water and relatively stable to change in pH.

2. Calcium channel blockers

These are substances which prevent the entry of calcium by blocking their transport channels across the membranes. These substances are either natural or synthetic in their origin. Among the natural blockers are lanthanum, manganese and vetatridine (Droogman et al., 1985). Among the synthetic are verapamil, D-600, nifidipine and ruthenium red (RR).

Lanthanum, a rare earth cation binds tightly to calcium carriers on the membrane. It causes inhibition of calcium transport by virtue of its very high affinity for the carriers and its consequent low rate of release to the internal phase (Reed and Bygrave, 1974a and 1974b).

Some synthetic chemicals like verapamil, D-600, nifidipine and RR acts as channel blockers.

Watson et al. (1971) showed that RR inhibits calcium transport in isolated mitochondria possibly by preventing either the uptake or recovery phase of the carrier. It is an inorganic dye and selectively inhibits calcium to transport and/or calcium to ATPase, without significantly affecting potassium, sodium or magnesium.

Verapamil: This is reported to block calcium transport from apoplast into the cytoplasm/cell. It is assumed to block voltage dependent calcium influx and therefore, cuts the supply of apoplastic calcium. But, it is less effective in blocking the receptor operated channels not involving membrane potential differences (Lehtonen,1984).

3. Calcium calmodulin inhibitor

Aluminum is also claimed as a calmodulin inhibitor. Siegel and Haug (1983) showed that aluminum interacts with calmodulin and inhibits its regulatory capacity.

Calmodulin inhibitor - chlorpromazine (CPZ) belongs to general group of compounds phenonthiazines or local anesthetics. This compound binds to the calmodulin protein in the presence of calcium and render its inactivity (Weiss and Levin, 1978). This has been used very widely to ascribe newer roles for calmodulin. In this context, it may be mentioned that (Elliott et al., 1983) using these inhibitors, in particular, CPZ and trifluoperazine (TPZ) has been implicated the involvement of calmodulin in a number of growth regulators mediated phenomenon like the GA induced - amylase activity in barley aleurone layers, IAA induced wheat coleoptile elongation, cytokinin induced betacyanin synthesis and increase in fresh weight in Amaranths tricolor cotyledons.

The involvement of calcium in the action of plant hormones shown by Elliott et al. (1983). In a detail analysis Raghothama et al. (1985) implicated the involvement of calcium on auxin induced elongation of oat and corn coleoptile segments by using three calmodulin antagonists with the phenothiazine structures. And he also showed auxin induced elongation completely inhibited in the presence of these drugs. These results shows possible involvement of calmodulin in auxin mediated elongation of coleoptiles. Many workers proposed a secondary messenger role for calcium in signal transduction mechanisms (Hepler and Wayne, 1985; Poovaiah and Reddy 1987). Calcium dependant phosphorylation also shown to occur in plants and this process was thought to play an important role in signal transduction amplification by regulating protein function (Veluthambi and poovaiah, 1984; Poovaiah and Reddy 1987). Auxin affects protein phosphorylation in etiolated pea segments (Reddy et al., 1987) and soybean nuclei (Reddy et al., 1988; Murray and Key, 1978). Poovaiah (1988) showed calcium involvement in auxin induced delay of leaf abscission in beans. These results indicate that calcium is involved in auxin induced responses in plant systems.

QUANTIFICATION OF PLANT GROWTH SUBSTANCES USING IMMUNOASSAY

Several endogenous hormones are involved in many steps which leads to the physiological processes. So,

accurate estimation of the levels of a particular hormone in a plant sample is essential. A number of sensitive analytical procedures have been used for indole-3-acetic acid (IAA) including gas chromatography mass spectroscopy (GC-MS) (Bridges et al., 1973), selected ion current monitoring (SCIM) (River and Pilet, 1974; Caruso et al., 1978), spectroflurimetric assays after conversion of IAA to the fluorescent indole- α - pyrone (Stoessl and Venis 1970) or using the fluorescent properties of IAA itself (Crozier et al., 1980). But their methods have limitations because of unstable nature of IAA. However, these methods can be used after extensive fractionation of plant material to remove impurities. It is time consuming and extremely expensive and also during fractionation there may be loss of hormones, which leads to low yields of IAA.

Bioassays, though sensitive enough to detect as little as 1 picogram of IAA (Nitsch and Nitsch, 1956) are non specific and of low accuracy. Thus, simple, rapid and inexpensive methods are needed for the quantitative detection and measurement of plant hormones in large samples of plant materials. So, there comes the importance of immunoassay techniques. This technique is sensitive and specific to detect physiological concentration of plant hormones even in crude samples. Immuno techniques have already been developed for the quantification of IAA, ABA, GAs, cytokinins and other plant growth regulators.

ESTIMATION OF AUXINS IN PLANT SAMPLES

Radioimmunoassay (RIA) provides a general method for measuring physiological concentration of hormones on a routine basis (Jaffe and Behrman, 1974). Weiler (1981) developed a radioimmunoassay for the detection of as little as 0.5 to 1 pmol indole-3-acetic acid in unpurified or partially purified plant extract. Pengully (1977) developed a specific radioimmunoassay for indole-3-acetic acid. Extracts of tobacco tissue were immunoassayed upto 0.2 ng to 12 ng range in partially purified samples.

A simple solid phase enzyme immunoassay was developed by Weiler et al. (1981) for the detection of as little as 3-4 pico grams of IAA. Using this assay, levels of IAA have been determined in coleoptiles of maize and oat. The distribution of IAA within single coleoptile was quantified and the production of IAA during the regeneration of physiological tip in Avena coleoptiles was investigated. And also the changes in levels of IAA and other major phytohormones were quantified during the growth of oat coleoptiles.

In the studies on testing for bulb quality, an enzyme linked immunosorbant assay was developed by Franssen et al. (1986) for detecting the endogenous quantity of IAA and ABA. IAA and ABA were conjugated to BSA by their carboxyl groups. Antibodies were raised in rabbits and a

good reproducible curve was developed. However, the standard curve for IAA was found to be less sensitive.

Hall (1990) developed monoclonal and polyclonal antibody against 2,4-D and picloram. And traces of 2,4-D and picloram contents were analyzed by using direct and indirect ELISA as well as radioimmunoassays.

AGRICULTURAL APPLICATION

Application of synthetic BR analogues in field and green house, increases the overall growth of the plants, as a result, yield increases.

BR EFFECT ON SEED GERMINATION

Yamaguchi et al. (1987) showed that very low concentration of BR (0.00001 ppm) increased the germination percentage of 13 year old seeds of rice cv. Norin No.22 in aseptic culture compared to control or seeds treated with ABA, NAA, GA_3 or zeatin. Han et al. (1988) also showed tobacco seeds treated with low concentration of epi-BR for 24 hours increased seed oxygen absorption, ethylene production and germination rate. Seed treatment also increased root dry weight and length. In 1988, Chang and Cai showed B. napus seeds cultured on Murashige and Skoog medium with 0.01 ppm BR gave better germination, larger cotyledons and higher root numbers. At low concentrations (0.0001 - 0.1 ppm) BR stimulated callus formation from cotyledon segments and rooting of

the callus. Also calluses from cotyledons cultured on media with 0.0001 - 0.001 ppm BR had a higher frequency of plantlet production.

VEGETABLE CROPS

In 1992, the US Department of Agriculture reported the biological effects of brassins on young bean plants and Siberian trees and found that brassins caused overall growth (Mitchell and Gregory, 1972). In field and greenhouse trials synthetic BR analogues significantly boosted the harvest of various vegetable and root crops. Eg: radish, lettuce, bean, pepper and potato (Maugh, 1981). Increase in yield of radish and lettuce to an extent of 15 to 30 per cent and 6 to 7 per cent increase in yield of bean and pepper plants in field trials was recorded but in green house study there was six times more yield compared to field study . Potato yield in small trials were shown to increase by 25 per cent.

Lim, (1985) showed BR increases the fresh weight, dry weight and number of tomato fruits and length, diameter, fresh weight and dry weight of pepper fruits compared to control.

Yield increases were reported when homo BR was applied to the flowers of tomato, cucumber and egg plants. This is mainly due to the increase in fruit setting of less viable flowers and subsequent growth promotion.

GRAIN CROPS

In Japan, homobrassinolide and brassinolide were tested for agricultural application in the last few years by several research groups and promising results were obtained. (Fujita, 1985; Takematsu et al., 1983 and 1985).

Han et al. 1988 showed foliar application of BR to tobacco increased photosynthesis and leaf chlorophyll content, leaf area and root dry weight, ATP and free amino acid content, phosphorous uptake, IAA synthesis, leaf nicotine, lipids, polyphenol, reducing sugar contents and tobacco yield and quality. Chen et al. (1990) also showed BR treatment at low concentration significantly increased number of rootlets on explants of tobacco and rootlet elongation and fresh weight of root system compared with the control. Krizek and Mandava (1983) also showed increase in chlorophyll content and significant increase in weight of primary leaves in bean plants.

Dogra and Thukrai (1989) showed significant increase in shoot length, number of leaves per plant and shoot dry weight in maize cv. Ganga by the application of steroids. By application of BR to wheat plants at different stages of development , the treatment from anthesis to maturity, increased ear yield by 15 per cent and increased grain yield by improving grain set and grain filling and also photosynthesis slightly. Hamada et al. (1985) also showed

similar results in wheat and maize. Shen et al. (1990) showed when maize seeds were soaked in 0.001 ppm BR and sown in pots subjected to drought or adequate water treatment, BR moderated the influence of water stress on growth. BR also reduced the effect of moisture stress on plasma membrane permeability, nitrate reductase activity, ATP, chlorophyll content and photosynthetic rate, resulting in increased grain yield. Braun and Wild (1984) showed increase in yield of mustard, and Gregory (1981) found yield increase in barley due to BR treatment.

Lim (1985) showed that at all levels of BR treatment, increased fresh weight, dry weight, leaf length and protein content in rice cultivars like Nong-Baik, Mil young 30 and Tai- Baik. In 1987, Wu et al. showed similar results in rice cv. Sang pang. BR treatment gives disease resistance and salt tolerance of rice (Hamada, 1986). In field trials application of 0.01 ppm BR at different growth stages promoted grain ripening (Hirai et al.,1991).

In Japan, (Fujita, 1985; Takematsu et al., 1983 and 1985) showed BR treatment gives protection or alleviation against environmental stress, such as injuries due to low temperature, herbicides, pathogenic micro organisms and salt.

FRUITS

Sugiyama and Kuraishi (1989) showed BR treatment resulted in higher fruit set in navel oranges but there were no differences in quality between treated and untreated fruits at harvest.

MATERIAL AND METHODS

III MATERIAL AND METHODS

Experiments were conducted to study the role of BR on growth in a few test systems. The reasons for growth of the tissue with BR were further looked into by studying the mode of action in excised wheat coleoptile tissue. Field experiments were conducted to study the productivity aspects of grapes and sunflower. The materials employed and the methods adopted are described in this chapter.

BRASSINOSTEROIDS: The chemicals brassinolide and homobrassinolide were supplied by Godrej Soaps Limited, Bombay. In few test system the effectiveness of both these compounds were tested and found that both the compounds were effective in similar concentration range (Rice leaf lamina inclination test). In most of the experiments particularly to find out the mode of action of brassinolide was used. Initial experiments conducted in this laboratory to study the relative effectiveness of brassinolide supplied by Godrej Soaps Limited, Bombay, with that of pure brassinolide from Sigma Co. U.S.A., indicated that the chemicals supplied by Godrej Soaps Limited is as effective as that of pure brassinolide in mung bean internode elongation bioassay (Prasad, personal communications).

3.1.1 WHEAT COLEOPTILE SEGMENT ELONGATION BIOASSAY

Wheat seeds (Triticum aestivum L. cv. Norin No.10) were surface sterilized in one per cent mercuric chloride for 10 minutes, then washed thoroughly with sterile distilled water, and soaked in distilled water for four hours. These seeds were sown at two cm depth in plastic trays containing saturated but thoroughly drained vermiculite and grown for 96 hours in dark at 25 ± 2 °C and 80 per cent relative humidity in seed germinator for 72 hours. Coleoptiles measuring 30 ± 2 mm length were selected and decapitated by removing 2-3 mm from the apex. Thirty minutes after removal of tip, 10 mm segment was obtained with a two bladed cutter just below the top cut end. The required number of sections were first floated on distilled water. Uniform 10 segments were collected, surface dried and placed in 50 mm diameter petri plates containing 5 ml of different concentrations of test solutions. These were incubated in dark. After 24 hours of growth, measurements were obtained by removing the sections from the petri plates. The segments were arranged end to end by lining on a wet glass plate and lengths were measured using scale with an accuracy of 0.25 mm.

3.1.2 MAIZE MESOCOTYL ELONGATION BIOASSAY

The procedure reported by Vanderhoef and Briggs (1978) was followed with a slight modification. The maize

kernels (Zea mays L. cv. Daccan) were soaked in distilled water and then sown at one cm depth in trays containing moist vermiculite,and germinated in complete darkness at 25 ± 2 °C and 90 per cent relative humidity in a germinator. After 96 hours of growth, the apical 20 mm of mesocotyl segments were cut and floated in different concentrations of test solutions in petri plates. Final lengths were determined by using scale with an accuracy of 0.25 mm.

3.1.3 SOYBEAN STEM SEGMENT ELONGATION BIOASSAY

Soybean (Glycine max L. cv. KBSH-1) seeds were sown in moist vermiculite and kept in dark for 10 days at 25 ± 2 °C and 90 per cent relative humidity. After 10 days of growth, 10 mm stem segments were excised and floated in different concentrations of test solutions and incubated in dark. After 24 hours of incubation, final length of segments were measured, and also recorded.

3.1.4 MUNG BEAN SEEDLING GROWTH BIOASSAY

Mung bean (Phaseolus aureus L.) seeds were surface sterilized in one per cent mercuric chloride for five minutes and then rinsed with distilled water. These seeds were germinated in petri plates containing wet filter paper, and incubated at 25 °C in seed germinator. After 2 days of incubation uniform pregerminated seedlings were placed in petri plates containing different concentrations

of BR (0.002 to 40 μM) and incubated in dark at 27 °C for 4 days. At the end of 96 hours, observations were recorded on hypocotyl length, root growth and hypocotyl thickness. The hypocotyl length and root growth were measured by a scale with an accuracy of 0.25 mm and hypocotyl thickness was measured using micrometer.

3.1.5 MUNG BEAN EPICOTYL ELONGATION BIOASSAY

Mung bean seeds were surface sterilized in one per cent mercuric chloride for five minutes, rinsed with distilled water and planted in moist vermiculite at a depth of 1.5 cm. Germination and growth of seedlings of first five days took place in ambient temperature and relative humidity. On the 5th day seedlings were removed from vermiculite and the root systems were excised. Five uniform length seedling shoots were selected and the cut end of the stem was immersed in a test tube containing three ml of different concentrations of test solutions and kept under diffused light for 48 hours. At the end of incubation period epicotyl lengths were determined.

3.1.6 CUCUMBER COTYLEDON EXPANSION BIOASSAY

The procedure used was that described by Udaya kumar and Krishna Sastry (1973). Seeds of cucumber, Cucumis sativa L. (Var. Guntur local) was germinated in total darkness for 48 hours at 28 °C. The seed coat was

removed, and 10-12 cotyledons were transferred to sterilized petri dishes (9 cm diameter) containing 3.5 ml of test solutions. All solutions were prepared in 2 mM phosphate buffer of pH 5.9. The petri dishes were exposed to weak fluorescent light (250 lux) for 3 days. They were then blotted and weighed.

3.1.7 COLEUS PETIOLE EXPLANT CURVATURE TEST

Vegetative shoots of coleus (Coleus blumei) having 4 to 6 pairs of leaves were selected for this experiment. Fourth or fifth node from apex with well developed leaves were separated and the leaf lamina was cut. Petioles measuring 3 cm were retained. About 2 cm stem segment below the two petioles were retained to facilitate planting over solid agar media. Stem segments with bud and two petioles present opposite to one another were planted in petri plates containing one per cent agar. Test solutions were applied with the help of a micropipette to the filter paper and punches placed in contact with freshly cut surface of the petiolar stump. Petri dishes were placed in a tray with a moist sheet of filter paper and maintained at 90 per cent relative humidity and temperature of 25±2 °C. After 72 hours of incubation change in the angle between the petioles was measured and recorded.

3.1.8 EXPANSION OF TOBACCO LEAF DISCS

Surface sterilized tobacco leaf discs measuring 8 mm in diameter were placed on MS agar medium (Murashige and Skoog, 1962) containing cytokinin at the rate of 0.25 $mg.l^{-1}$ with different concentrations of test compounds in 100 ml Erlenmeyer flask in an inoculation hood aseptically. These flasks were incubated at 24±3 °C under fluorescent light. Light period was maintained throughout the experiment. Observations were recorded on fresh weight and diameter of leaf discs on 20^{th} day of inoculation.

3.1.9 POLLEN GERMINATION TEST IN *Leucina leucocephala*.

Pollen grains of subabul were incubated in 100 µl of pollen germination media (PGM - 30 mg $ca(No_3)_2$, 20 mg KNo_3, 20 mg $MgSo_4$, 10 mg H_3Bo_3 and 3 g sucrose in 100 ml of double distilled water) in cavity slides. Pollen grains in the media were maintained in a humid chamber at 25 °C. Percentage of germination and pollen tube length were measured from 10 minutes after incubation to 30 minutes at 5 minutes interval under microscope. Tube length was measured in microns using ocular and stage micrometer.

3.1.10 RICE LEAF LAMINA INCLINATION BIOASSAY

Uniform sized seeds of rice (*Oryza sativa* L. cv.Tan-gin-bozu) was surface sterilized with 0.5 per cent mercuric chloride for 5 minutes and seeds were then

repeatedly washed using sterile distilled water and soaked for 2 hours. Seeds were germinated in petri dishes over filter paper soaked with distilled water. When the radicle was 2 mm in length, five uniform germinated seeds were planted over 0.5 per cent agar taken in petri dishes of 50 mm diameter and incubated in diffused light for further seven days in growth chamber maintained at 25 °C and 90 per cent relative humidity. Seven days after planting on agar medium a drop of 10 µl of either water or test solution (BR or HBR) was placed on ligule of the second leaf and maintained for further 2 days under diffused light.

To know the effect of other hormones, the following compounds and the concentrations mentioned below are used.

Phytohormones	**Concentration range used**		
Auxin-IAA	0.0176 µg	to	1.76 µg
Gibberellin-GA	0.0342 µg	to	3.42 µg
Ethylene-Ethephon	0.01 µg	to	1.0 µg
Cytokinin-BA	0.2 ng	to	0.2 µg
BRs-Brassinolide	0.1 µg	to	0.5 µg
Abscisic acid	0.0264 µg	to	2.64 µg

For excised leaf segment test, Rice leaf segments consisting of one cm long leaf lamina, ligule and one cm long leaf sheath were floated on different concentrations of BR (0.0002 to 2 µM). Two days after application of test solution, the angle of inclination was measured.

3.2 INTERACTIONS OF BR WITH AUXINS AND OTHER HORMONES IN A FEW TEST SYSTEMS

In many test systems, BR evoked responses similar to that of IAA. Based on this information a few experiments were conducted to see the interaction effect between BR and Auxins in different bioassay systems.

3.2.1 INTERACTION OF BR WITH IAA IN THE ELONGATION OF WHEAT COLEOPTILE SEGMENTS

Wheat coleoptile segments were incubated in small petri plates (50 mm) containing 5 ml of different concentrations of BR (0.002 to 2 μM) with or without IAA (1 or 10 μM). At the end of 24 hours of dark incubation period, the final length of coleoptile segments were determined as mentioned earlier.

3.2.1.1 INFLUENCE OF SEQUENTIAL TREATMENT OF BR AND IAA ON GROWTH OF COLEOPTILE SEGMENTS

In this experiment, the influence of sequential treatments of IAA and BR on coleoptile tissues were studied to know further about the interaction effect of IAA and BR.

Coleoptile segments were pretreated with BR for different durations (1, 2, 3 and 4 hours). Then segments were washed in distilled water and transferred to IAA for post treatment for a further duration of 24 hours. Final lengths were measured after 24 hours of dark incubation in IAA.

Keeping 3 hours as optimum duration for pretreatment, further experiments were carried out. Coleoptile segments were pretreated in 10 ml of BR for 3 hours and then transferred to IAA for post treatment. Final lengths of segments were measured at the end of incubation period.

In another set of experiment, the sequence of treatment was reversed by giving IAA for 3 hours and then transferred to 0.2 µM BR for a duration of 24 hours.

To study whether the pretreatment effect of BR is stable, the coleoptile segments were pretreated with 0.2 µM BR followed by floating on distilled water for 2 hours. Then, segments were post treated with IAA for 24 hours. Final lengths were measured after 24 hours of dark incubation in IAA.

3.2.2 INTERACTION OF BR WITH IAA ON RICE LEAF LAMINA INCLINATION TEST

To study the interaction effect of BR and IAA on rice leaf lamina inclination, different concentrations of IAA were mixed with a known concentration of BR and the interaction effect was assessed.

3.2.3 INTERACTION OF AUXIN (NAA) WITH BR IN TOBACCO LEAF DISC EXPANSION TEST

Surface sterilized tobacco leaf discs were placed on MS agar medium containing cytokinin at the rate of 0.25 $mg.l^{-1}$ with different concentrations of BR (0.02 to 2.0

μM) or NAA (2.7 to 10.8 μM) or different concentration combinations of BR + NAA. These flasks were incubated at 24 ± 3 °C under fluorescent light. After 20 days of incubation observations were recorded on fresh weight and diameter of the leaf discs.

3.2.4 INTERACTION OF BR AND CYTOKININS IN CUCUMBER COTYLEDON EXPANSION BIOASSAY

Cucumber cotyledons were excised as mentioned earlier. The cotyledons were placed in 5 ml test solution containing different concentrations of BR (0.002 to 20 μM) with or without BA or BA +KCl and kept under diffused light for 4 days. Fresh weight of the cotyledons were determined at the end of the incubation period.

3.3 COMPARISON OF GROWTH PATTERN OF BR WITH IAA

Since BR was showing similar effects as that of IAA, in this set of experiments, BR induced growth pattern was compared with that of IAA.

3.3.1 INFLUENCE OF IAA AND BR ON GROWTH PATTERNS OF WHEAT COLEOPTILES

Wheat coleoptiles were raised and prepared as mentioned earlier. Ten segments were incubated in water, IAA and BR with three replications. In the first set of experiment, increase in length was recorded initially at every 10 minutes interval for 2 hours using a dissecting

microscope. After 2 hours, observations were recorded at every 30 minutes interval.

In second set of experiment, observations were recorded at every one hour interval till 4 hours then at 3 hours interval upto 24 hours.

3.3.2 EFFECT OF IAA AND BR ON ACIDIFICATION OF THE INCUBATION MEDIA

Fifty coleoptile segments of 10 mm length were incubated in 5 ml of water, BR or IAA and BR +IAA in small beakers in dark. pH of the incubation media and growth of coleoptile were recorded at every 30 minutes interval till two hours. The relationship between IAA and BR induced acidification of incubation media and growth was plotted.

3.3.3 EFFECT OF INHIBITORS ON IAA AND BR INDUCING GROWTH OF WHEAT COLEOPTILE SEGMENTS

In a series of experiments the mode of action of BR in inducing cell elongation in wheat coleoptile segments was studied. In all these experiments the BR effect was compared with that of IAA.

3.3.3.1 EFFECT OF GALACTOSE ON IAA AND BR INDUCED COLEOPTILE GROWTH

Wheat coleoptile sections were incubated in petri plates containing 5 ml of 0.5 μM potassium phosphate buffer pH 6.0 with 10 mM sucrose and different

concentrations of galactose with or without IAA or BR. Incubation of these sections was carried out in dark for 24 hours at 25 °C.

In another experiment wheat coleoptile segments were preincubated for 90 minutes in 0.5 mM potassium phosphate buffer pH 6.0, 10 mM sucrose and galactose then transferred to IAA or BR and incubated in dark. IAA or BR induced growth was determined at one hour interval upto 7 hours and final growth was measured at the end of 24 hours of incubation in dark.

3.3.3.2 EFFECT OF METABOLIC INHIBITORS ON IAA OR BR INDUCED GROWTH OF COLEOPTILE SEGMENTS

Effect of KCN on IAA and BR induced growth : Coleoptile segments were incubated in different concentrations of KCN with or without IAA and BR. At the end of 24 hours incubation period, the final length of coleoptile segments were measured as mentioned earlier.

Effect of DCCD (Dicyclohexylcarbodiimide) on IAA and BR induced growth: Wheat coleoptile segments were incubated in different concentrations of DCCD with or without IAA or BR in dark at 25 ± 2 °C for 24 hours. At the end of 24 hours of incubation the final length of coleoptile segments were measured.

In another experiment increase in length was measured at every 15 minutes interval upto 3 hours and then at 30

minutes interval upto 8 hours and the final reading after 24 hours of incubation.

3.3.3.3. EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON IAA AND BR INDUCED ELONGATION OF WHEAT COLEOPTILE SEGMENTS

Coleoptile segments were incubated in petri plates containing 5 ml of different concentrations (1000 to 0.1 μM) of protein synthesis inhibitor, cycloheximide or 5-Fluorouracil or Chloramphenicol with or without IAA or BR. Final length of coleoptiles were measured 24 hours after incubation.

To study the pretreatment effect, coleoptiles were pretreated with cycloheximide (10 μM) and 5-Fluorouracil(10 μM) for 2 and 4 hours. After 2 and 4 hours, coleoptiles were transferred to IAA or BR with 5-Fluorouracil or cycloheximide for the post treatment of 24 hours. Final lengths of coleoptile segments were measured at the end of 24 hours incubation period.

3.3.4 INFLUENCE OF ADENOSINE PHOSPHATES ON IAA AND BR INDUCED GROWTH IN COLEOPTILE SEGMENTS

To study the effect of source of energy in the form of adenosine phosphates, coleoptiles were incubated in different concentrations of AMP, ADP and ATP in dark for 24 hours. Final length was measured at the end of incubation period.

For pretreatment experiment, coleoptiles were pretreated with ATP for 30 minutes and 60 minutes and then transferred to water, IAA and BR for post treatment. Final length was measured at the end of 24 hours of incubation in dark.

3.3.5 THE ROLE OF BR ON AUXIN BIOSYNTHESIS IN COLEOPTILE SEGMENTS

Since BR mimics the response of IAA in many test systems, it can be assumed that BR might be increasing endogenous auxin concentration which leads to elongation growth. To test this, coleoptile tip segments including the apices were cut under dim red light. These segments were incubated in 1 mM potassium phosphate buffer containing tryptophan and other cofactors like pyrodoxyl phosphate (10 μM) and a keto acid (α-ketoglutaric acid 10 μM) for conversion of tryptophan to IAA. The influence of GA and BR on conversion of tryptophan to IAA was studied. Dimedone an inhibitor of indole acetaldehyde to IAA, was also used in some treatments to know the role of BR in auxin biosynthesis.

3.3.6 ESTIMATION OF IAA IN COLEOPTILE TISSUE BY IMMUNOASSAY TECHNIQUE

3.3.6.1 SYNTHESIS OF IMMUNOGENIC PROTEIN - HORMONE CONJUGATE

Plant hormones being low molecular weight cannot induce an immunogenic response by themselves. To make

them immunogenic, Haptens are coupled with macromolecular carrier, preferably a protein. To raise antibody against IAA, IAA-BSA conjugate was synthesized.

3.3.6.2 SYNTHESIS OF IAA - BOVINE SERUM ALBUMIN (BSA) CONJUGATE

This was performed according to the method of Weiler (1981). The synthesis was carried out under nitrogen in dark. 52.3 mg of recrystallized IAA was dissolved in 2 ml dimethyl formamide (DMF). To this 75 µl tri-n-butylamine was added and the solution cooled to -15 °C. After addition of 40 µl isobutyl chlorocarbonate, the formation of the mixed anhydride was allowed to proceed for 8 minutes. After the formation of the mixed anhydride (8 minutes reaction time at -15 °C), 421 mg of BSA dissolved in 22 ml of an equal mixture by volume of DMF and water and 0.42 ml 1 M NaOH was added to the reaction mixture by stirring. Mixture was allowed to stand at 0 °C for one hour. Another 0.2 ml of 1 M NaOH was added and stirring was continued for 5 hours. The reaction mixture was then dialyzed two times against 10 per cent DMF and finally for 4 days against distilled water, lyophilized and stored at -18 °C.

3.3.6.3 CALCULATION OF COUPLING RATIO OF IAA TO PROTEIN

Principle: The number of molecules of IAA bound to a molecule of BSA gives the binding ratio and was calculated from the differences in spectra of BSA and

IAA-BSA conjugate. The absorption of the hapten bound to BSA was determined at a wave length of 266 nm. The absorption of solution of BSA alone at the same concentration (1 $mg.ml^{-1}$) was also taken at the same wave length. From the concentration of the ligand (moles) and the concentration of BSA (moles) present in the conjugate, the binding ratio was determined.

3.3.6.4 IMMUNIZATION

The choice of animal: The selected species for raising antibody was rabbit. Individual variation in response taken care by immunizing at least three animals.

Immunization schedule: Antisera were raised in New Zealand rabbits using intradermal injection of 1.5 mg conjugate in 0.5 ml saline mixed with 0.5 ml Freunds complete adjuvant (FCA).

After one week, a repeat injection of 1.5 mg conjugate in 0.5 ml saline mixed with 0.5 ml incomplete adjuvant, was given. One week after this, a booster injection was given followed by a second booster at the rate of 1 mg conjugate in incomplete adjuvant five weeks later.

Collection and storage of antiserum: Seven days after the last booster injection given, approximately 30 ml blood was collected from an incision of the marginal ear vein

in a clean dry glass bottles. It was kept at room temperature for two hours and then over night at 4 °C. The serum was decanted and centrifuged at 3500 rpm for 10 minutes to remove residual red cells and crude serum was stored at -20 °C.

3.3.6.5 QUALITATIVE AND QUANTITATIVE ANALYSIS OF SERUM FOR ANTIBODIES

For qualitative analysis, immunochemical technique - immunodiffusion was used. Quantitative analysis of the antiserum for specific IgG was done by the quantitative precipitation analysis (QPA). The specificity of IAA-BSA IgG towards the hapten (IAA) was tested by the membrane binding assay using labelled IAA.

3.3.6.5.1 Immunodiffusion

Principle: The basic principle involved in immunochemical techniques is that a specific antigen will combine with its specific antibody to give an antigen-antibody complex. This complex is usually insoluble and can be seen with naked eye.

Materials and reagents required:

1. Phosphate buffer saline (PBS) - pH 7.4, 0.1 M :

   A. $Na_2HPO_4.2H_2O$ - 17.6 $gm.l^{-1}$

   B. $NaHPO_4.2H_2O$ - 15.6 $gm.l^{-1}$

PBS pH 7.4, 0.1 M - 40.5 ml of A + 9.5 ml of B made up to 100 ml

2. One per cent agar prepared in PBS 7.4 pH, 0.1 M containing 0.02 per cent sodium azide and 0.87 per cent NaCl.

3. 0.87 per cent saline

4. Staining solution - Acetic acid - 10 ml
Methanol - 50 ml
Distilled water - 50 ml
Comasie brilliant blue - 0.22 mg

5. Destaining solution - Acetic acid - 7 ml
Methanol - 43 ml
Distilled water - 50 ml

Procedure: Agar gels were made in petri dishes approximately 4 mm in thickness, required number of wells 4 mm apart were punched from edge to edge.

In the central well, crude antiserum was placed. In the surrounding wells different concentrations of BSA or IAA-BSA conjugates were added. The petri dishes were incubated in desiccator (under humid conditions). The wells were observed and refilled with appropriate solutions daily. When the precipitation bands became visible, the gel was washed in 0.87 per cent saline for 12 hours with frequent changes of the solution. It was dried over a glass plate by using hair drier by keeping a filter paper layer. Then gel was put in staining solution for 10 minutes and then destained by keeping the stained gel in destaining solution for half an hour to one hour. The gel was kept for permanent record.

3.3.6.5.2 Quantitative precipitation analysis

Principle: If increasing amounts of an antigen are mixed with aliquots of suitable antibody solution a precipitate forms in some of the tubes. This precipitate is called as antigen-antibody precipitate. After equilibration, the antigen-antibody precipitate may be isolated from the supernatant sample by centrifugation and may be estimated with a suitable protein assay. A typical precipitant curve has three zones.

i) Zone of antibody excess ;
ii) Zone of equivalence;
iii) Zone of antigen excess

In the zone of equivalence, maximum antigen-antibody precipitate is formed provided the zone of equivalence can be determined, the precipitin reaction provides a very accurate method for quantitative assay for antigens.

The procedure of Humayun and Jacob (1973) was followed. Details of the technique are given below.

Reagents: 1. Tris-HCl buffered saline (pH 7.0)
2. Antiserum
3. BSA (1 $mg.ml^{-1}$)
4. IAA -BSA conjugate.

Procedure: Quantitative precipitation reactions were performed with 50 µl of antiserum and various quantities of IAA - BSA conjugate and in buffer in a total volume of 0.5 ml. The tubes were incubated at 37 °C for four hours

and subsequently left at 4 °C for 72 hours with occasional dispersal of settled precipitate. The precipitate was centrifuged at 2000 rpm for 15 minutes at 4 °C and drained the supernatant with tissue paper. The precipitates were resuspended in 0.5 ml buffer at 4 °C and the centrifugation repeated. The washing procedure was repeated twice. The precipitate after the last centrifugation was dissolved in 0.01 M NaOH and the protein was estimated quantitatively.

3.3.6.5.3 Membrane-binding assay (Humayun and Jacob, 1973)

Principle: The assay is a modification of the hapten inhibition test, which uses the specificity of the antigen-antibody reaction to elucidate the chemical nature of antigenic determinants. This assay is commonly used to test the extent of cross reaction of the antiserum with related antigens which sometimes share a common determinant with the antigen of interest. Thus, a whole range of antigens are screened to give evidence on the chemical nature of the antigen, and the specificity of the IgG.

Reagents: 1. 0.01M Tris-HCl buffered saline (pH 7.5) with 0.02 per cent azide.

2. ^{14}C IAA (0.1 ml contains 5000 cpm)

3. Antiserum.

4. Nitrocellulose membranes.

Procedure: Membrane-binding assay carried out with sera, labelled IAA and diluted with cold buffer containing 0.02 per cent azide. All reactions were carried out in a total volume of 0.3 ml, containing 0.1 ml of diluted antiserum, 0.1 ml of the radioactive IAA solution and 0.1 ml of buffer. The antiserum was added last. And in blanks 0.1 ml of normal rabbit serum substituted for the antiserum. After incubation at 37 °C for 30 minutes, the contents were filtered on a prewetted membrane (by filtering 0.7 ml of buffer for 2 minutes with the help of millipore glass filter holders. Filtration was done at a rapid rate (4-5 $ml.min^{-1}$). The tubes were rinsed with 0.5 ml of buffer. After filtration, the membranes were transferred into scintillation vials, dried at 100 °C for 10 to 15 minutes, cooled and then measured the radioactivity after adding 2 ml of 0.5 per cent PPO in toluene.

3.3.6.6 HAPTEN CARRIER PROTEIN CONJUGATE

In indirect ELISA, antigen (hapten) is coated to the wells. As mentioned earlier, haptens will not bind to the polystrene surface of microtiter plate by themselves. Therefore, IAA is bonded chemically to an inert protein such as casein instead of BSA to avoid reactivity with non-specific antibodies to BSA that are present in the serum. Casein was chosen based on the results of the dot-blot test.

DOT BLOT TECHNIQUE

Principle: The dot immunobinding assay is a sensitive system which enables characterization and identification of an immobilized antigen by the use of antibody probes. This can subsequently be visualized by ratio labelled or enzyme conjugated secondary antibodies. In dot immuno bolt, antigen or different proteins are immobilized on graded nitro cellulose membranes.

Objective: This technique was used to probe the cross reaction of antisera raised against IAA-BSA with other proteins.

Materials required:

1. Nitrocellulose membrane.
2. Different concentrations of different proteins (casein, ovalbumin and gelatine).
3. Blocking solution (1% casein in PBS 10 mM pH 7.0)
4. Primary antibody (crude serum)
5. Labelled anti immuno globulin (secondary antibody conjugated with alkaline phosphatase); i.e., ALP Goat-anti rabbit I
6. Substrate solution:

3M Tris pH 8.8	-	0.67 ml
NaCl	-	0.117 g
1 mM $MgCl_2$	-	100 ml
BCIP (5-Bromo-chloro 3 - Indolyl PO_4 (dissolved in 200 µl of DMF)	-	2.00 mg
NBT (Nitro blue tetrasolium)	-	6.00 mg

(dissolved in 200 µl of DMF + 20 µl of water)
Volume made upto 20 ml.

7. Phosphate buffer saline (pH 7.4):

Na_2HPO_4 - 1.44 g

$K_2H_2PO_4$ - 0.24 g

Volume made up to 1 litre.

Procedure: Three microlitres of different concentrations of casein, gelatin and ovalbumin were applied separately and dried on the nitro cellulose membrane, limiting the binding of the antigen in a small area. Other binding sites were saturated with blocking agent by keeping the membrane strips in the blocking solution and were shaken gently for 16 hours. Then, they were washed thoroughly with PBS and were put in 1:500 dilutions of crude primary antibody for two hours. After washing, the membranes were put in 10 ml of secondary antibody (1:1500 dilutions) for one hour. They were rinsed thrice in PBS and the membranes were put in 10 ml of the substrate solution.

The formation of blue colour indicates cross reaction (Plate 1). The intensity of the colour is often used as a basis of the degree of cross reaction with antibody.

PREPARATION OF IAA-CASEIN CONJUGATE

IAA has conjugated with casein similar to IAA-BSA conjugation procedure mentioned earlier.

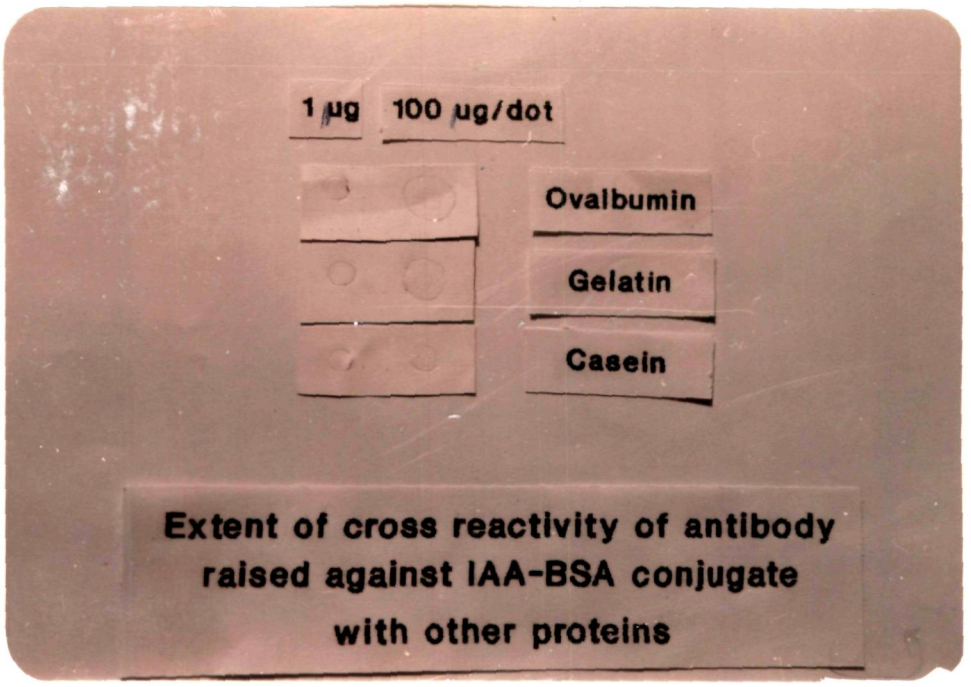

1 µg
100 µg/dot
Ovalbumin
Gelatin
Casein
Extent of cross reactivity of antibody raised against IAA-BSA conjugate with other proteins

Plate 1:

ENZYME IMMUNOASSAY

Enzyme immunoassays (EIA) are based on two important biological phenomenon:(i) the extraordinary discriminatory power of antibodies, based on the ability of the immune system of vertebrates to produce a virtually unlimited variety of proteins (antibodies) each with an affinity for a specific foreign compound (antigen or hapten); and (ii) the extremely high catalytic power and specificity of enzymes, which may quite often be detectable with great ease. EIA consists of a two-pronged strategy: the reaction between the immuno reactants (antibody with the corresponding antigen) and the detection of that reaction using enzymes, to the reactants, as indicators. A plethora of such other labels have been employed, such as radioactive tracers (radioimmunoassay, RIA), chemiluminescent and fluorescent labels. ELISA may be used for assaying antigens by either a competitive method or a double antibody method and for assaying a specific antibody by an indirect method. All these methods require the preparation of a calibration curve during the assay.

3.3.6.7 INDIRECT ELISA

Principle: In this method, labelled anti-I (secondary antibody conjugated with alkaline phosphate) are allowed to bind with primary antibodies (raised against IAA) complexed to antigen (IAA-casein) attached to a solid

phase. After the complex has been washed with buffer, the enzyme substrate was added and the enzyme activity measured by reading the colour developed using ELISA reader.

Reagents:

1. Hapten carrier protein conjugate (IAA - casein)
2. Primary antibodies (Antibody raised against IAA-BSA conjugate)
3. Secondary antibody: Goat anti-rabbit I conjugated with alkaline phosphatase, SIGMA
4. Coating buffer (0.05 M, pH 9.5):

   Na_2CO_3 - 1.59 g

   $NaHCO_3$ - 2.93 g

   NaN_3 - 0.2 g

   H_2O - 1 litre

5. Washing buffer (PBS with tween pH 7.4):

   NaCl - 8 g

   KCl - 0.2 g

   KH_2PO_4 - 0.2 g

   Na_2HPO_4 - 1.15 g

   Tween 20 - 0.5 ml

   H_2O - 1 litre.

6. Dilution buffer (blocking buffer pH 7.4):

   Casein - 1 g

   Washing buffer - 100 ml

PROCEDURE:

1. Coating the ELISA plate with antigen (IAA-Casein): Antigen was diluted using coating buffer (100 ng/200 µl), and 200 µl of it was added to each well. The plate was incubated at 4 °C over night. After the incubation, the contents were discarded (Fig. 3a) and washed thrice with washing buffer at three minutes interval each time.

2. Blocking the non-specific sites in the wells: The wells were filled with blocking buffer and the plate was incubated for 90 minutes at 37 °C. Then the wells were washed thrice with washing buffer as mentioned above.

3. Addition of primary antibody: Primary antibody of 200 µl diluted with dilution buffer was added to the wells and the plates were incubated at 37 °C for three hours to allow the IAA antibodies to bind to the IAA molecules bound to each well (Fig. 3b). The wells were washed thrice with washing buffer as explained earlier.

4. Addition of secondary antibody: Secondary antibody (Goat- anti-rabbit antibody) of 200 µl diluted 1:1500 times with dilution buffer, was added to the wells. The plate was incubated at 37 °C for two hours. The second antibody attaches to the IAA antibodies (Fig. 3c) and washed thrice with washing buffer.

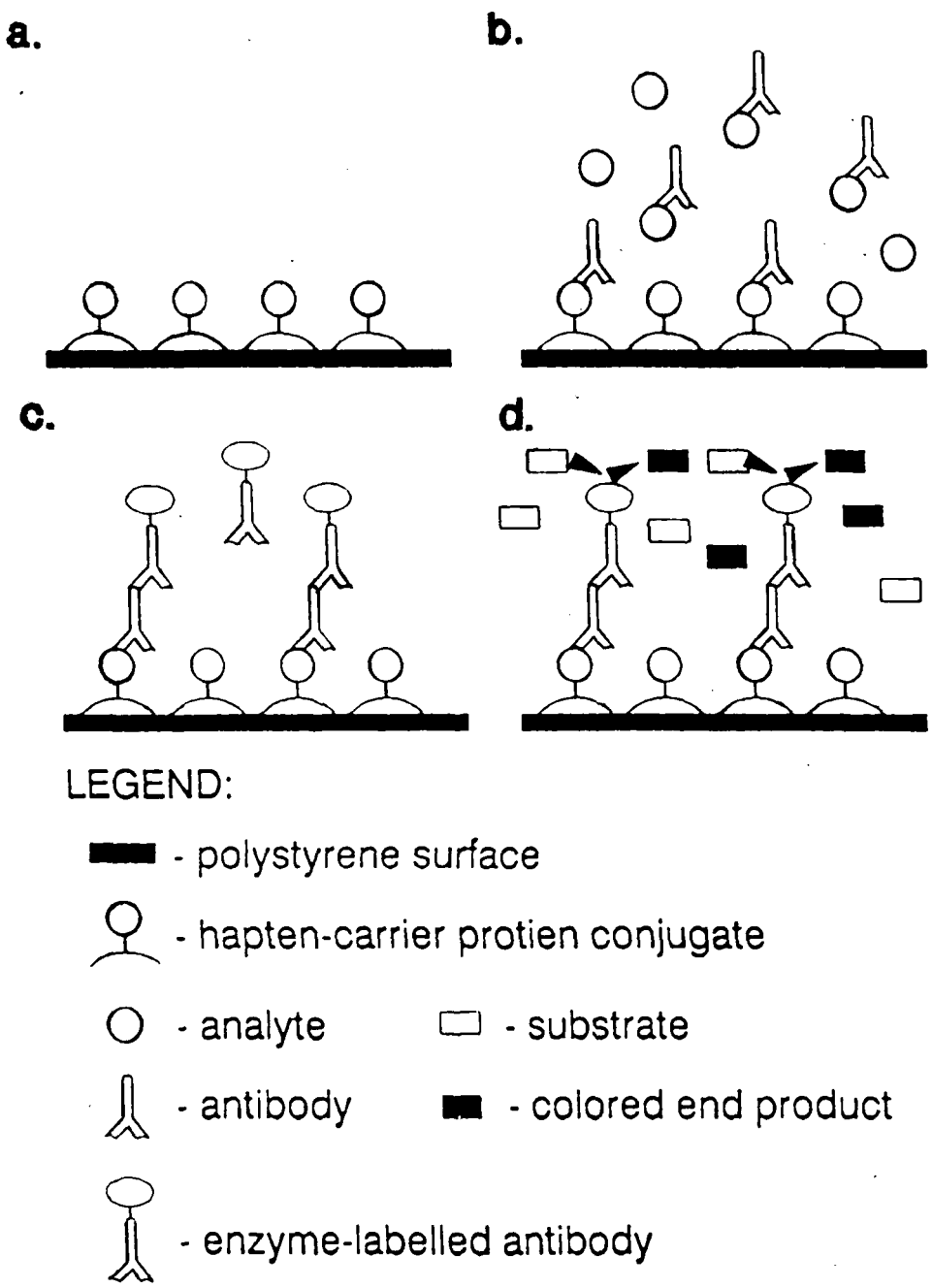


Fig. 3: Indirect ELISA. a) A polystyrene surface is coated with hapten linked to carrier protein. b)Immobilized hapten and analyte compete for binding to antibody. c) A second antibody labelled with an enzyme binds to the first antibody. d) Substrate for the enzyme is added resulting in a coloured end product which is measured spectrophotometrically.

5. Detection: The substrate for alkaline phosphatase, para nitrophenol phosphate (PNPP) was prepared in coating buffer without azide (1 $mg.ml^{-1}$) and 200 µl was added to each well. The plate was incubated at 37 °C for colour development (15 minutes) (Fig. 3d). Reaction was stopped by adding 25 µl of 5N KOH. PNPP added to two empty wells was taken as control. The absorbance was read at 405 nm using ELISA reader.

3.3.6.7.1 CONSTRUCTION OF STANDARD CURVE

1. IAA - Casein conjugate
2. Primary antibody
3. IAA standards (working range 0 - 1000 pmoles)
   (0, 0.02, 2, 20, 50, 100, 200, 400, 600, 800 and 1000 pmoles

   Preparation of IAA stock (5 mM solution)
   a. 8.8 mg of IAA in 10 ml dimethylformamide (DMF)
   b. Dilute this to 1:50 times with DMF to have 0.1 mM stock solution
   c. 0 to 1000 pmoles IAA solutions were prepared from 0.1 mM stock solution by using TBS pH 7.5
4. Secondary antibody (Goat-anti rabbit antibodies)
5. Substrate (PNPP)
6. Buffers:
   a. Coating buffer
   b. Dilution buffer/Blocking buffer
   c. Washing buffer.
   d. Tris buffer saline (TBS) (7.5 pH)
      Trizma base - 3.03 g
      NaCl - 5.84 g
      $MgCl_2.6H_2O$ - 0.20 g
      Volume made up to 1 l

Procedure: In step 3, instead of 200 µl of diluted serum, the IAA antibody was mixed with a different concentration of free IAA diluted with TBS buffer, before being added to the coated microtiter well. The free IAA will bind to the specific antibodies in solution and prevent their subsequent binding by competition inhibition to the IAA/casein attached to the plate. To this, 200 µl of diluted secondary antibody to which the chromogenic enzyme is attached, was added to the wells, which binds to the IAA antibody. Thus, the intensity of the final colour in each well is inversely proportional to the concentration of free IAA added to the well. That is, the more free IAA that is added, the less intense is the colour reaction.

Calculations:

1. Optical densities were recorded.
2. Average of optical densities of duplicate standards was take
3. Per cent binding of each standard was calculated using the formula,

$$\text{Per cent binding} = \frac{\text{Standard OD - ZB OD}}{B_o \text{ OD - ZB OD}} \times 100$$

   B_o = 100 % binding

   ZB = zero % binding

4. Per cent binding was converted to logit B/B_o
   Logit B/B_o = Ln $[(B/B_o\ \%)\ /(100-B/B_o)]$
5. Standard curve was drawn using IAA (pmoles. 0.1 ml^{-1}) against logit B/B_o values.

3.3.6.8 EXTRACTION PROCEDURE FOR IAA ESTIMATION BY IMMUNOASSAY (Weiler, 1981)

Coleoptile tip segments (10 mm) were incubated in tryptophan and other cofactors with and without BR, GA which is known to induce conversion of tryptophan to IAA.This was also included as one of the treatments. At different intervals after incubating the coleoptile tissue in the medium, the coleoptile segments were extracted in ice-chilled 80 % methanol and methanol was evaporated under vaeum to leave an aqueous aliquots. The concentration of IAA in the tissue was estimated using immunoassay technique.

3.3.7 INFLUENCE OF BR ON EXTENT OF BINDING OF ^{14}C IAA TO MEMBRANES AND AUXIN BINDING PROTEINS (ABPs)

This experiment was conducted with radioactive IAA (^{14}C IAA) to see the influence of BR on binding of IAA to membranes and auxin binding proteins on the membranes.

3.3.7.1 Extraction of cytosolic and membrane proteins:

Extraction buffer: 0.1 M Tris-HCl pH 8.0

50 ml 0.1 M Tris + 29.2 ml of 0.1 M HCl and make up the volume to 100 ml.

2 % SDS buffer: Tris HCl 0.1 M pH 8.0

2 g SDS

5 g Mercaptaethanol

Composition of Brays solution :

Naphthalene	-	6 g
PPO	-	400 mg
POPOP	-	20 mg
Methanol	-	10 ml
1,4 Dioxan	-	used to make up the volume to 100 ml

PPO - 2,5 Diphenyloxazole

POPOP - 1,4 bis [2-(5-Phenyloxazolyl)] - Benzene phenyl - oxazolylphenyl - orazolyl phenyl

Procedure: Ten mm length wheat coleoptiles were treated with ^{14}C with IAA or BR or IAA + BR and incubated in dark at 25 ± 2 °C for 12 hours and also 24 hours. At the end of incubation period, coleoptiles were removed and washed in water thoroughly. The coleoptiles were ground in extraction buffer pH 8.0 at 4 °C. Ground material was centrifuged at 12,000 rpm at 4 °C for 10 minutes. The supernatant contains cytosolic proteins. Thus obtained pellets were ground in 2 per cent SDS buffer, pH 8.0 and centrifuged at 12,000 rpm at room temperature for 10 minutes. The supernatant was precipitated by 8 volumes of cold acetone and centrifuged at 15,000 rpm at room temperature for 10 minutes. The radioactivity in the precipitated membrane proteins and cytosolic fraction, were assessed using liquid scintillation system. Known quantity of protein fraction was transferred to small filter paper discs and after drying, they were dropped in

2 ml Bray's solution and the radioactivity was determined by LSS-20.

3.3.7.2 Extraction of ABPs

Buffers:

Homogenization medium (pH 8.0): Sucrose - 250 mM
EDTA - 0.1 mM
$MgCl_2$ - 0.1 mM
Tris - 50 mM

Wash Media: Sucrose - 250 mM
$MgCl_2$ - 0.5 mM
Sodium citrate - 10 mM
Citric acid - 10 mM

Assay medium (pH 5.5): Sucrose - 250 mM
$MgCl_2$ - 5.0 mM
Sodium citrate - 10 mM
Citric acid - 10 mM

Standard Scintillant: Toluene with 0.5 % POP
Toluene 100 ml 0.5 gm of POP

Raising plant material: Wheat coleoptiles were raised as mentioned earlier. Ten mm length coleoptile segments were taken and the primary leaves were removed from the coleoptiles and kept in beaker over chilled ice.

Particle preparation: All operations were carried out at 0 to 2 °C. Coleoptiles were chopped thoroughly in an

equal weight of homogenization medium using stainless steel razor blades and then macerated in chilled pestle and mortar. This brei was filtered by squeezing through nylon cloth, the residue was ground, in the same amount of homogenization medium, and filtered through nylon cloth. The combined filtrates were recentrifuged for 10 minutes at 6800 g, which removed most of nuclei, plastids and mitochondria. The supernatant was further centrifuged for 20 minutes at 1,33,000 g. The pellets were resuspended by stirring with a glass rod in a wash medium using about one half the volume of the original homogenate. Again the particles were pelleted by centrifugation for 20 minutes at 1,33,000 g and pellets were resuspended in the assay medium. Usually of such a volume as to contain particles from 0.5 g of tissue, fresh weight of suspension or 1 mg ABP in 2 µl of assay medium.

Binding assay: Labelled IAA (0.2 ml of 3000 cpm) was added to assay medium containing 5 mg of particular protein/ml, and held in ice for 30 minutes. Sub-samples of 2 ml were dispensed into polypropylene centrifuge tubes in ice, which contained 20 µl of ethanol, either alone or containing unlabelled IAA (0.2 ml of 1000 µM) and held in ice for 30 minutes. Samples containing plain ethanol (sample A) and 1000 µM IAA (sample B). The tubes were centrifuged for 20 minutes at 1,33,000 g at 0 °C. The supernatants were decanted, the tubes were allowed

to drain for about 10 minutes by placing upside down on a filter paper. Then, 1 ml of methanol was placed in each tube. After one hour, which was sufficient to extract all radioactivity from the pellet, the methanol was transferred to a scintillation vial for determination using toluene with 0.5 per cent PPO and the radioactivity was measured using LS-400 scintillation spectrometer. Radioactivity of sample B is termed non-specific binding and that of A minus B is termed specific binding.

A - B = ^{14}C IAA Specific binding

A: Fraction contains ^{14}C IAA bound to specific and non-specific sites

B: Fraction contains only ^{14}C IAA bound to non-specific sites.

3.4 ROLE OF CALCIUM IN BR AND IAA INDUCED GROWTH

In this set of experiments, the role of calcium in BR and IAA induced elongation of wheat coleoptile was studied by altering the endogenous calcium levels by using substances which reduces active calcium concentration in the cytosol like EGTA, CPZ, RR, lanthanum chloride and verapamil. Three sets of experiments were conducted in this study.

1. Effect of different concentrations of calcium inhibitors on IAA and BR induced growth in wheat coleoptile segments.

2. Pretreatment effect of calcium inhibitors on IAA and BR induced growth in wheat coleoptile segments.
3. Effect of calcium inhibitors on IAA and BR induced growth in normal and calcium enriched wheat coleoptile segments.

3.4.1 RAISING AND PREPARATION OF NORMAL AND CALCIUM ENRICHED COLEOPTILES

Normal coleoptiles were raised as mentioned earlier. For calcium enriched coleoptiles, wheat seeds were soaked in one per cent calcium chloride solution for 4 hours and sown at 2 cm depth in plastic trays containing vermiculite saturated with 1 per cent calcium chloride, but thoroughly drained, and grown for 96 hours in the dark at 25 ± 2 °C and 80 per cent relative humidity in seed germinator. Coleoptile sections were prepared as mentioned earlier.

3.4.1.1 Estimation of calcium concentration in tissue

Known number of calcium enriched coleoptiles were ground and calcium was estimated by Flame Photometry.

3.4.2 CALCIUM INHIBITORS ON IAA AND BR INDUCED GROWTH IN WHEAT COLEOPTILES

Coleoptile segments were incubated in different concentrations of EGTA, CPZ, verapamil, lanthanum chloride and RR with and without IAA. Ten coleoptile segments were selected and placed in small petri plates containing

5 ml of the test solutions and incubated in dark for 24 hours at 25 ± 2 °C temperature. The final length was measured as mentioned earlier.

3.4.3 PRETREATMENT EFFECT OF CALCIUM INHIBITORS ON IAA AND BR INDUCED GROWTH IN COLEOPTILE SEGMENTS

The coleoptile segments were pre incubated in water, EGTA, CPZ, RR, lanthanum chloride or verapamil for 4 hours. At the end of 4 hours of incubation, sections were rinsed in distilled water, surface dried and then transferred to water, IAA or BR. Sections were incubated in dark at 25 ± 2 °C for 24 hours. At the end of incubation period, increase in length was measured.

3.4.4 INFLUENCE OF CALCIUM INHIBITORS ON IAA AND BR INDUCED GROWTH IN NORMAL AND CALCIUM ENRICHED COLEOPTILE SEGMENTS

Normal and calcium enriched coleoptiles were floated with or without calcium inhibitors like EGTA, CPZ, RR and Verapamil in the presence of IAA or BR. These segments were incubated in dark for 24 hours at 25 ± 2 °C. The final length was measured at the end of incubation period.

3.4.5 INFLUENCE OF ETHYLENE INHIBITORS ON RICE LEAF LAMINA INCLINATION

Rice seedlings were raised as mentioned earlier. Ten ul of water or solution of STS (5 μM) and cobalt chloride (500 μM) were placed on the ligule of second leaf lamina of eight day old seedlings and maintained for 24 hours at

90 per cent relative humidity in a glass tub lined with wet filter paper. After 24 hours, 10 µl of BR (0.02 µM) was placed in same place namely, ligule and maintained for another 2 days. Leaf bending was recorded by measuring angle between leaf lamina and leaf sheath at the end of incubation period.

3.4.6 ROLE OF CALCIUM ON BR INDUCED LEAF LAMINA INCLINATION

The role of calcium on BR induced leaf lamina inclination was tested by using calcium sequestering agent EGTA and calcium channel blockers like RR and verapamil.

The second leaf ligule was pretreated with 10 µl of EGTA, RR and verapamil for 24 hours. After 24 hours of pretreatment, BR was applied to the ligule and maintained for 48 hours. The angle of inclination was measured at the end of incubation period.

3.5 INFLUENCE OF BR ON GROWTH AND PRODUCTIVITY

In this set of experiments the influence of BR on growth and yield parameters were assessed.

3.5.1 INFLUENCE OF BR ON LEAF AREA DEVELOPMENT

In this set of experiment, the influence of BR on leaf area development in young seedlings of horsegram (Dolichos biflorus) and rice was studied.

Horsegram leaf expansion : Horsegram plants were raised in small plastic bowls with 2 kg soil and plants were thinned to retain only eight plants per pot. When the plants were 10 days old, the cotyledonary leaves of the plants were smeared with known amount of BR, BA and GA. The influence of BR treatment on leaf area of first trifoliate leaf produced by the plant was determined on 4th and 14th day after application of BR. Internodal elongation of seedlings was also determined on 6th and 9th day after application of BR, BA and GA.

Rice leaf expansion: Rice seeds cv. Tan-gin-bozu was soaked in water for two hours and then surface sterilized in 0.5 per cent mercuric chloride solution. Seeds were then repeatedly washed using sterile distilled water and germinated in petri dishes over filter paper soaked with sterile distilled water. When radicle was 2 mm in length five uniformly germinated seeds were planted over 0.5 per cent agar gel in petri plates and incubated in diffused light for further 5 days in growth chamber maintained at 25 °C and 90 per cent relative humidity. When the first leaf emerged and reached its maximum size, a drop of 10 µl of either water or IAA (10 µM) or BR(10 ppm) alone and in combination was placed on the ligule and maintained for further two days under diffused light. After three days, leaf lamina expansion was recorded by measuring the leaf area of the leaf which received BR as well as, the

new leaf which was produced above the leaf which received the treatment.

3.5.2 INFLUENCE OF BR ON GROWTH AND PRODUCTIVITY OF GRAPES

In many test systems, BR induces cell elongation and also cell expansion. With an objective to use this property in increasing productivity, the influence of BR on berry growth in grapes was evaluated in three field experiments.

Three experiments were conducted to evaluate the influence of BR and GA alone and in combination on bunch weight of grapes. Among them two field experiments were conducted on the variety Anab-E-Sahi and other on the variety Dilkush.

In all the three experiments, the individual clusters (30 replications) of immature fruits were dipped (1st dip) 20 days after flowering and 2nd dip 10 days after 1st dip in different concentrations of BR alone and along with 25 ppm GA solutions. Improvement in fruit size and quality parameters were studied at maturity.

3.5.3 INFLUENCE OF BR ON GROWTH AND PRODUCTIVITY OF SUNFLOWER

The influence of foliar application of BR on growth and productivity of sunflower crop was studied in a field experiment in two seasons. The crop was raised under

protected irrigated conditions, following all the package of practices recommended for the crop.

Field experiment was conducted under irrigated conditions and the crop was irrigated once in a week throughout the crop growth period.

Layout : RBD
Variety : Hybrid KBSH - 1
Replication : 5
Treatments : 5
1. Control
2. Foliar spray of BR 1.0 ppm
3. Foliar spray of BR 2.0 ppm
4. Foliar spray of BR 5.0 ppm
5. Foliar spray of BR 10.0 ppm

The chemical was applied to give a thorough wetting to the foliage.

Stage of application of the chemical: In first season, 20, 40 and 60 days after sowing, the crop was harvested on 95th day and weight of seeds per earhead was calculated. In addition to this, seed yield per square meter land area was also determined.

In the second season, spray was given at 30th and 45th day after germination. The crop was harvested on 95th day after sowing and the observations on biomass accumulated in various organs of the plant and seed yield were recorded.

EXPERIMENTAL RESULTS

IV RESULTS

In the present study the influence of Brassinosteroids (BRs) on several growth and development aspects were investigated. The results obtained from these investigations are given in this chapter under the following broad headings:

1. Influence of Brassinosteroids on the growth responses in test systems,
2. Interaction of BR with auxins and other hormones on growth responses in a few test systems,
3. Mode of action of BR in induction of growth responses, and
4. Agricultural application of BRs : Influence of Brassinolide on growth and productivity in sunflower and grapes.

4.1 INFLUENCE OF BRASSINOSTEROIDS ON THE GROWTH RESPONSES IN TEST SYSTEMS

Many research groups have shown that Brassinosteroids promote dual responses in several plant tissues (Mitchell et al., 1967; and Worley and Mitchell, 1971). Namely, cell elongation and cell division. In the present investigation, experiments were conducted to study the role of BRs in inducing elongation or expansion growth in few test systems. In some instances, the Brassinosteroid effect was compared with the effect of other hormones in well defined test systems. This was performed to investigate the relative effectiveness of BR over other hormones.

4.1.1 WHEAT COLEOPTILE SEGMENT ELONGATION BIOASSAY

Comparative effects of BR and IAA on the elongation of wheat coleoptile segments were studied (Fig. 4a and 4b).

The results indicated that both IAA as well as BR were effective in inducing elongation of coleoptile segments. However, IAA induced coleoptile segment growth was more than that of BR. The optimum concentrations of IAA and BR to induce maximum elongation were 10 and 0.2 µM, respectively. Coleoptile segments incubated in 10 µM IAA elicited an elongation of nearly 45 per cent more than that of the control. The elongation elicited by optimum concentration of BR was about 36 per cent over control.

4.1.2 MAIZE MESOCOTYL ELONGATION BIOASSAY

The influence of different concentrations of BR and IAA on Maize mesocotyl segments elongation was studied. Ten mm mesocotyl segments were prepared from four day old maize seedlings grown in dark and floated over different concentration of BR and IAA. The final length of segments was measured after 24 hours of incubation in dark (Fig. 5a and 5b).

The results indicate a linear increase in mesocotyl segment elongation with increasing concentration of IAA from 0.01 µM to 1 mM. However, elongation elicited by BR was observed 0.002 to 20 µM concentration. At optimum concentration, mesocotyl segment elongation increased by

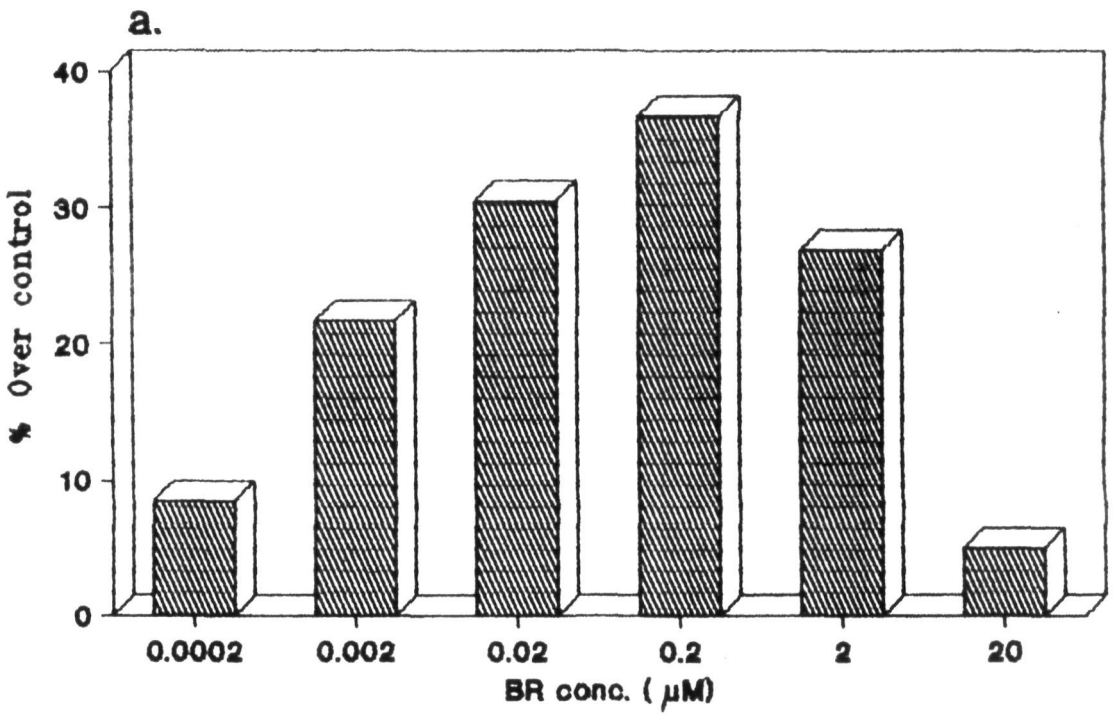


Fig. 4: Influence of different concentrations of a) BR, and b) IAA on elongation of wheat coleoptile segments.

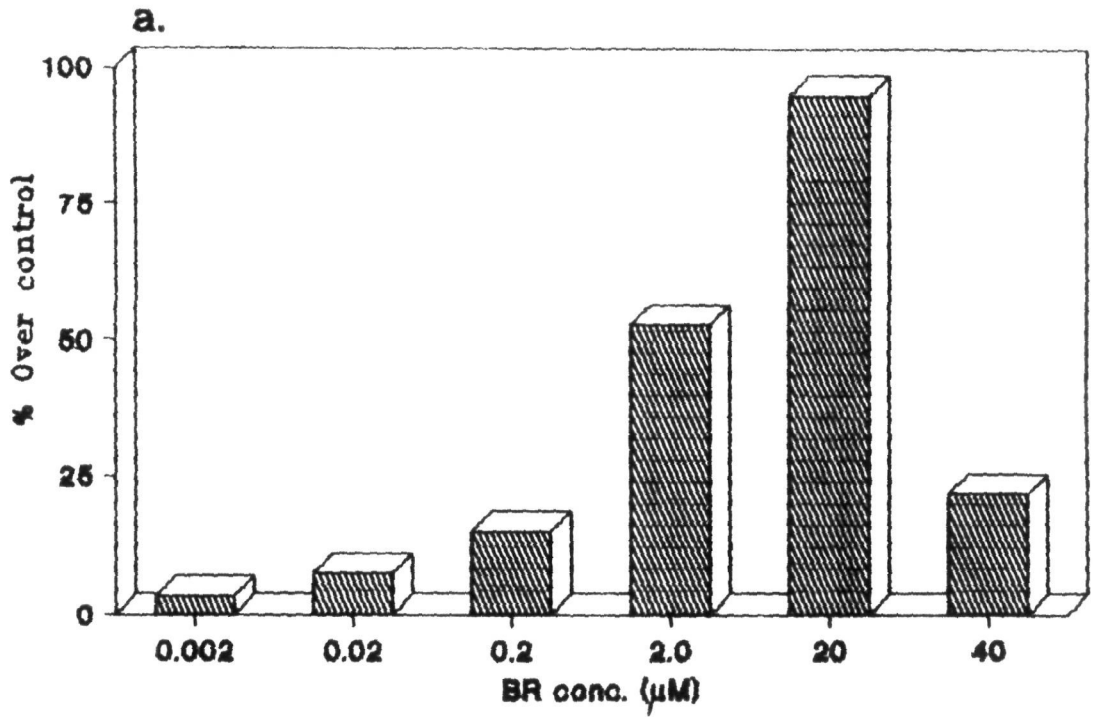


Fig. 5: Influence of different concentrations of a) BR, and b) IAA on elongation of maize mesocotyl segments.

162 per cent with IAA and 86 per cent with BR over control. Maximum growth of mesocotyl segments was observed at concentration of 20 μM BR and 10 μM IAA.

4.1.3 SOYBEAN STEM SEGMENT ELONGATION BIOASSAY

From ten day old soybean seedlings, 12 mm stem sections were prepared from the first internode and floated over different concentration of BR and IAA. IAA and BR dependent increase in length of soybean stem section is shown in Fig. 6a and 6b. Presence of 0.01 μM IAA during the 24 hour incubation period caused a 65 per cent increase in length over control. The increase in length was linear from 0.01 μM to 1 mM concentration and at 1 mM IAA the increase in length was 100 per cent more than that of control.

BR at low concentration (0.002 μM) caused a small increase in elongation. However, with a concentration of 0.2 μM, maximum elongation was recorded. A further increase in BR concentration in the medium reduced per cent increase in elongation. At a concentration of 40 μM, the BR dependent response is almost completely reduced. Optimum concentration for maximum elongation was 1 mM for IAA and 0.2 μM for BR. At 0.2 μM of BR, the increase was 160 per cent over control compared to 100 per cent by 1 mM of IAA.

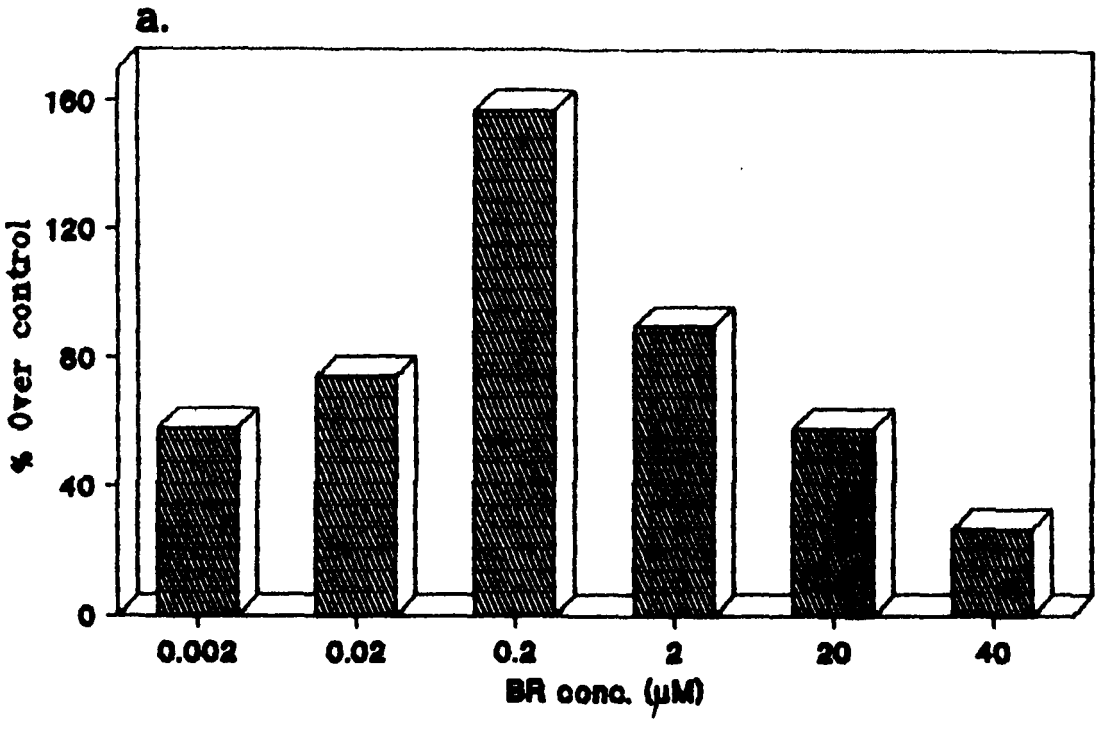


Fig. 6: Influence of different concentrations of a) BR, and b) IAA on elongation of soybean stem segments.

4.1.4 MUNG BEAN SEEDLING GROWTH BIOASSAY

In this experiment, pregerminated mung bean seeds were incubated in petri dishes containing different concentrations of BR. This is done to study the influence of BR on elongation of hypocotyl, radial expansion of the stem, and also on root growth in intact seedlings. Observations were recorded at the end of 96 hours of incubation period.

Results indicated that BR at 0.2 μM concentration and above significantly inhibited elongation of hypocotyl. This effect increased linearly with increasing concentrations of BR up to 2 μM. With further increase in the concentration of BR there was a drastic reduction in stem elongation, and at 40 μM concentration elongation was almost completely prevented(Fig.7a).

Elongation of mung bean seedling roots were not altered until 0.2 μM concentration of BR. At 2 μM there was 22 per cent reduction in root growth and the inhibition increased upto 40 μM. At 40 μM concentration of BR, the elongation of root was almost prevented (Fig. 7b).

The concentration effect of BR on radial expansion of mung bean hypocotyl in intact seedling system is shown in Fig. 7c The influence of BR concentrations from 0.002 to 0.2 μM on radial expansion of hypocotyl was marginal. Stem diameter was significantly increased at BR

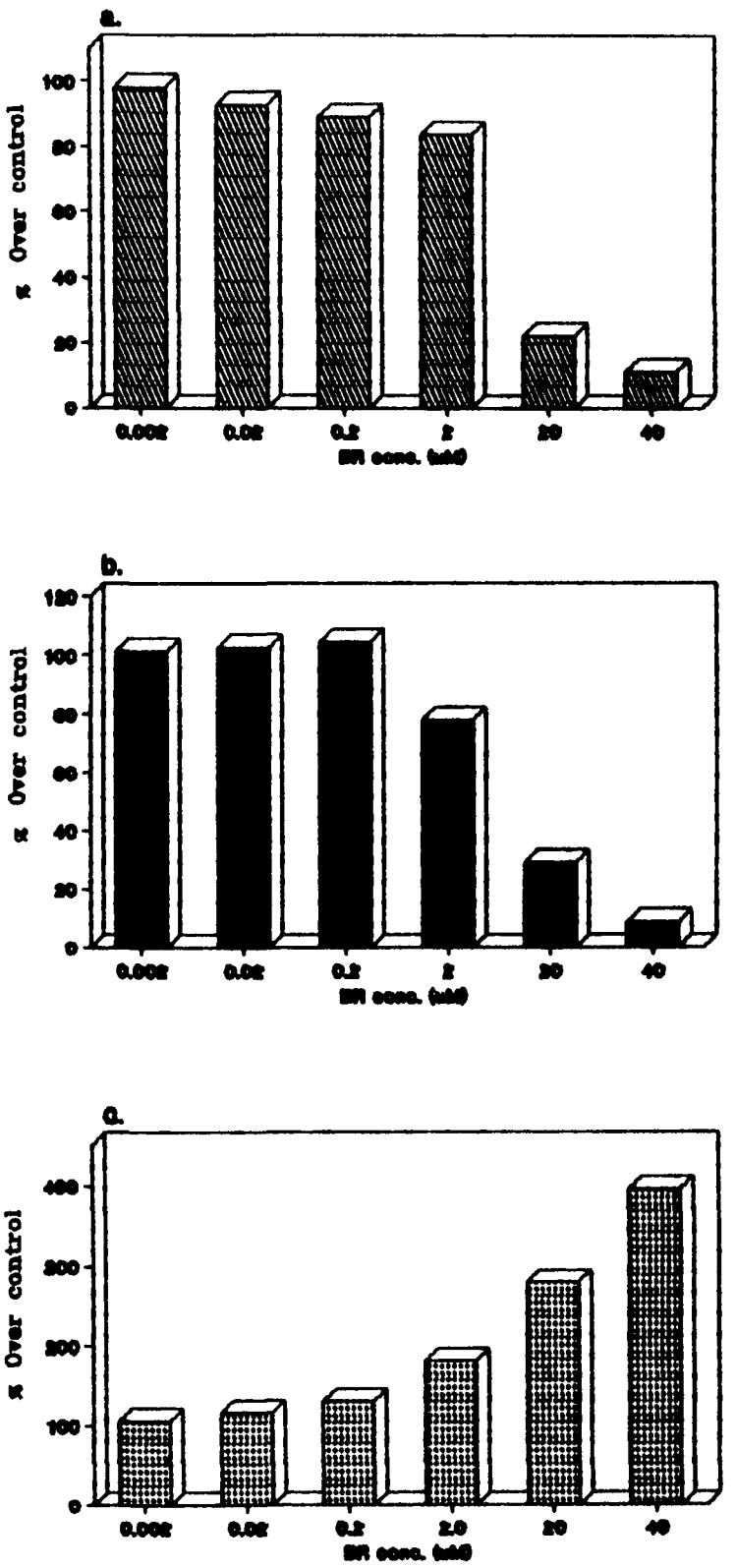


Fig. 7: Influence of different concentrations of BR on mung bean seedling growth.
a) hypocotyl elongation,
b) root elongation, and
c) radial hypocotyl expansion.

concentrations from 2 μM to 40 μM. At BR concentration of 40 μM radial expansion of the hypocotyl increased by 195 per cent over control.

4.1.5 MUNG BEAN EPICOTYL ELONGATION BIOASSAY

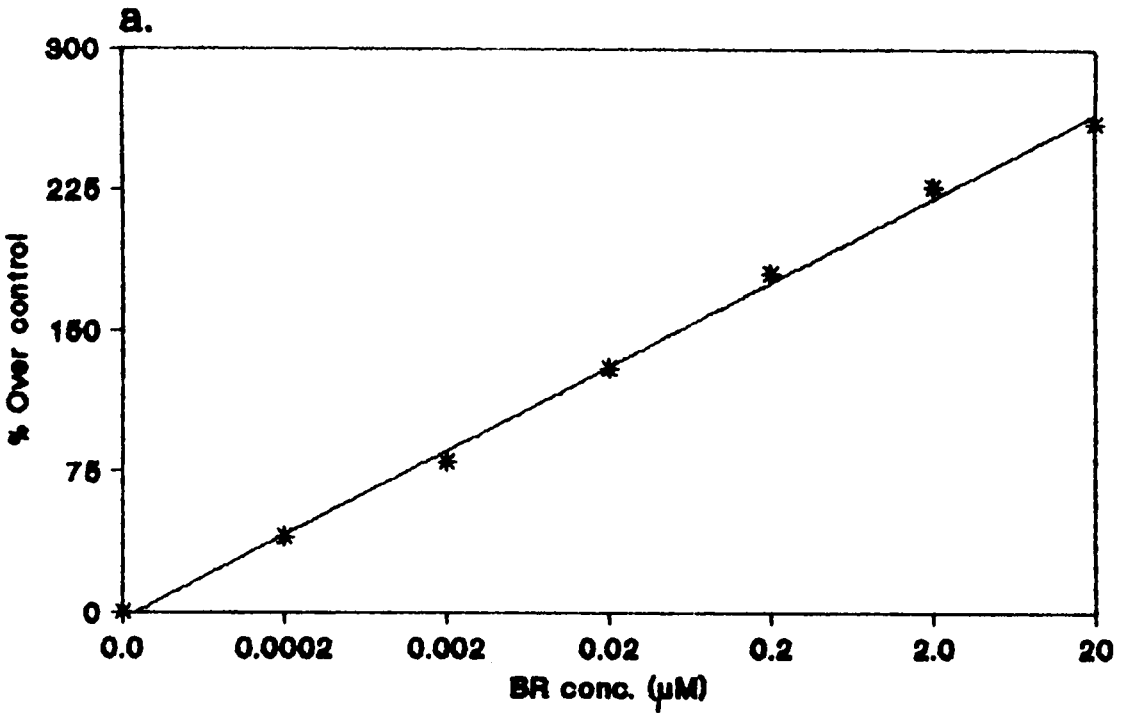
The influence of BR in the concentration range of 0.0002 μM to 20 μM on mung bean epicotyl elongation was studied and was compared with that of gibberellic acid over the concentration range of 0.01 μM to 10 mM. The epicotyl elongation as a function of concentrations of BR and GA is shown in Fig. 8a and 8b.

The response of mung bean epicotyl to exogenous application of BR was evaluated at concentration range from 0.0002 to 20 μM. BR induced elongation was observed even at the lowest concentration of 0.0002 μM. The response increased with increasing concentration of BR upto 20 μM.

At concentration range from 0.01 μM to 10 mM, gibberellic acid elicited a linear increase in elongation growth of mung bean epicotyl. Among the concentrations of gibberellic acid studied, maximum response to an extent of 367 per cent over control was observed at a concentration of 10 mM over control.

4.1.6 CUCUMBER COTYLEDON EXPANSION BIOASSAY

In this test system, the influence of cytokinin and BR on expansion of excised cucumber cotyledons was



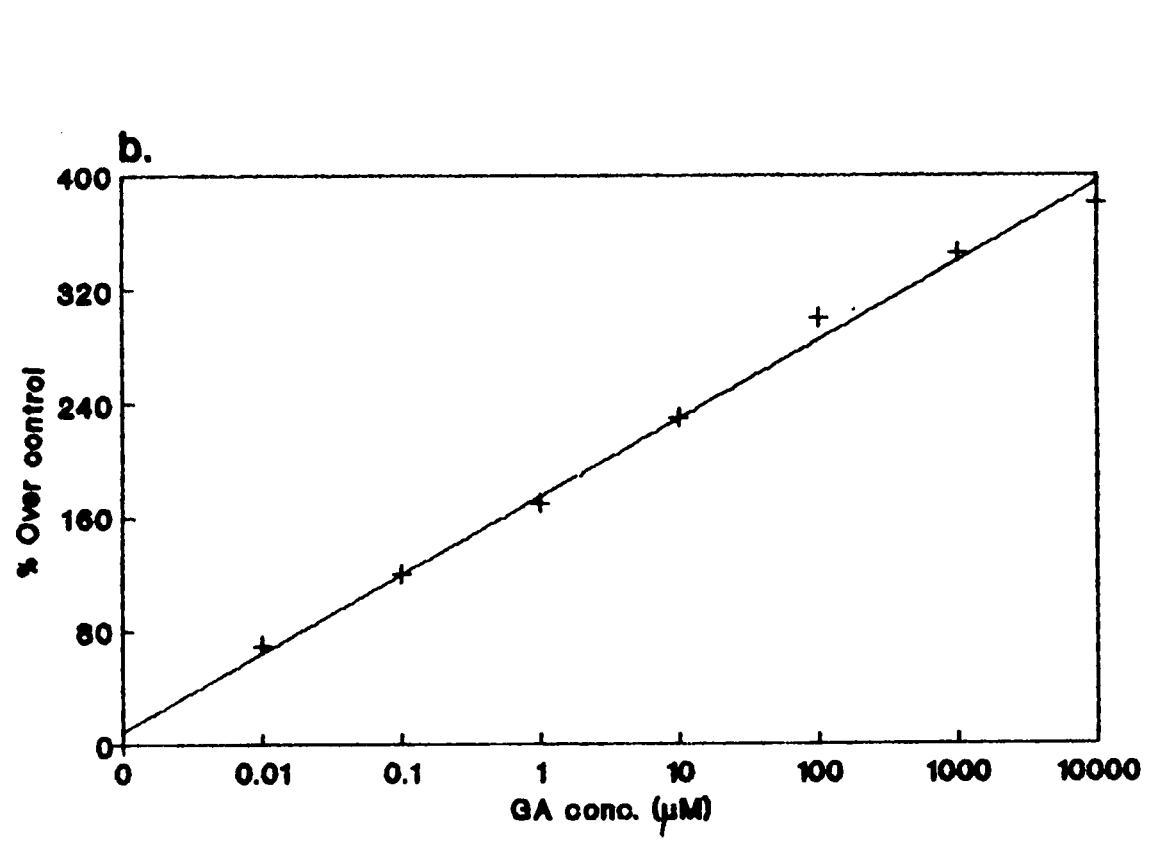


Fig. 8: Influence of different concentrations of a) BR, and b) GA on mung bean epicotyl elongation.

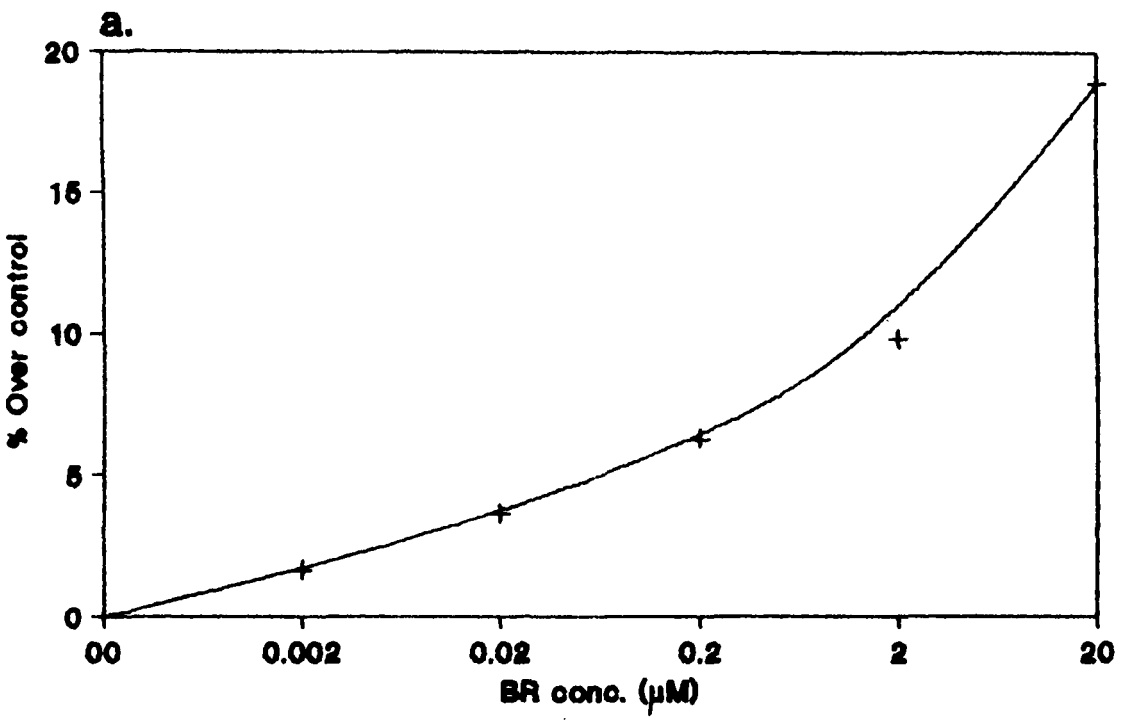
investigated (Fig. 9a). BR evoked poor response in this system. BR at concentration of 0.002 μM and 0.02 μM was ineffective in increasing the fresh weight of cotyledons, and in the range of 0.2 to 20 μM, resulted a linear increase in fresh weight of the cotyledons. Maximum increase in fresh weight to an extent of 19 per cent was observed at 20 μM BR.

A cytokinin, Benzyl Adenine (BA) was highly effective in evoking fresh weight increase in cucumber cotyledons as shown in Fig.9b. The increase in fresh weight of cotyledons was proportional to the logarthmic concentration of BA. BA at a concentration of 6.5 μM induced an increase of 162 per cent in fresh weight of the cotyledon over control.

4.1.7 COLEUS PETIOLE EXPLANT CURVATURE TEST

The angle of curvature developed between the two petioles present opposite to one another in response to different concentrations of BR was measured.

In this experiment, BR activity was measured in terms of change in the angle between the two opposite petioles in coleus (Fig. 10). Exogenous application of BR to cut ends of petioles decreased the angle. The angle between the petioles reduced as BR concentration increased from 0.002 μM to 40 μM. The reduction in angle ranged from 16 per cent at 0.002 μM to 89 per cent at 40 μM. In this



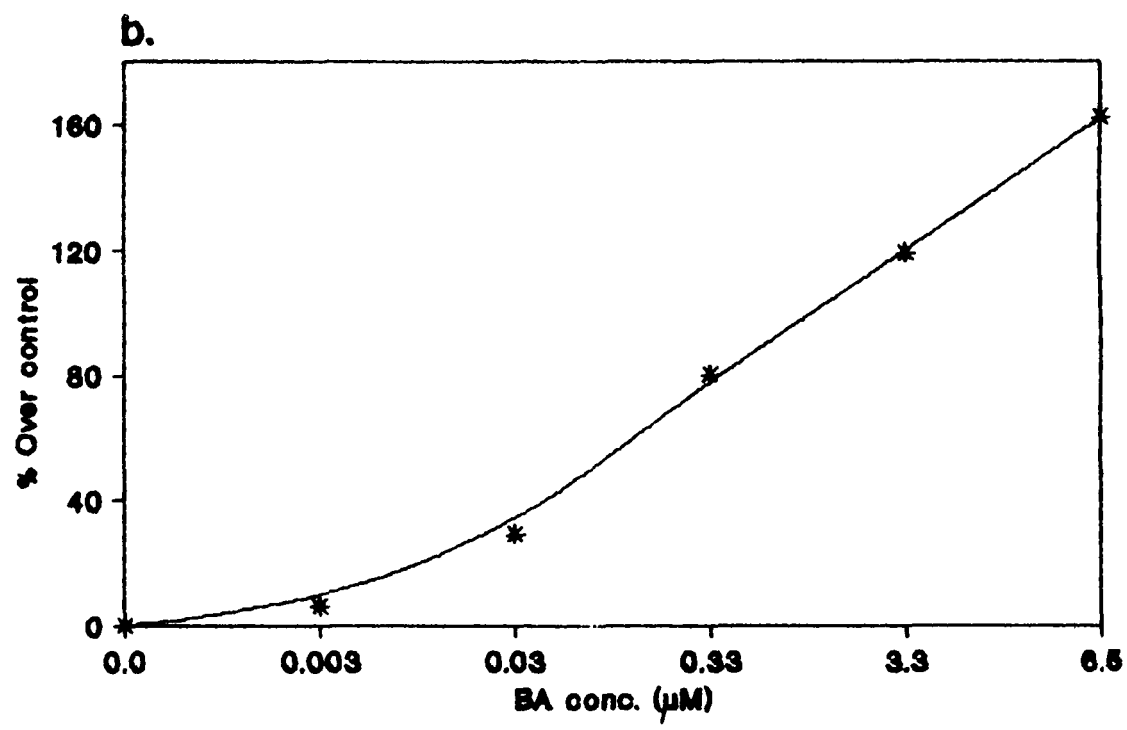


Fig. 9: Influence of different concentrations of a) BR, and b) BA on fresh weight of cucumber cotyledons.

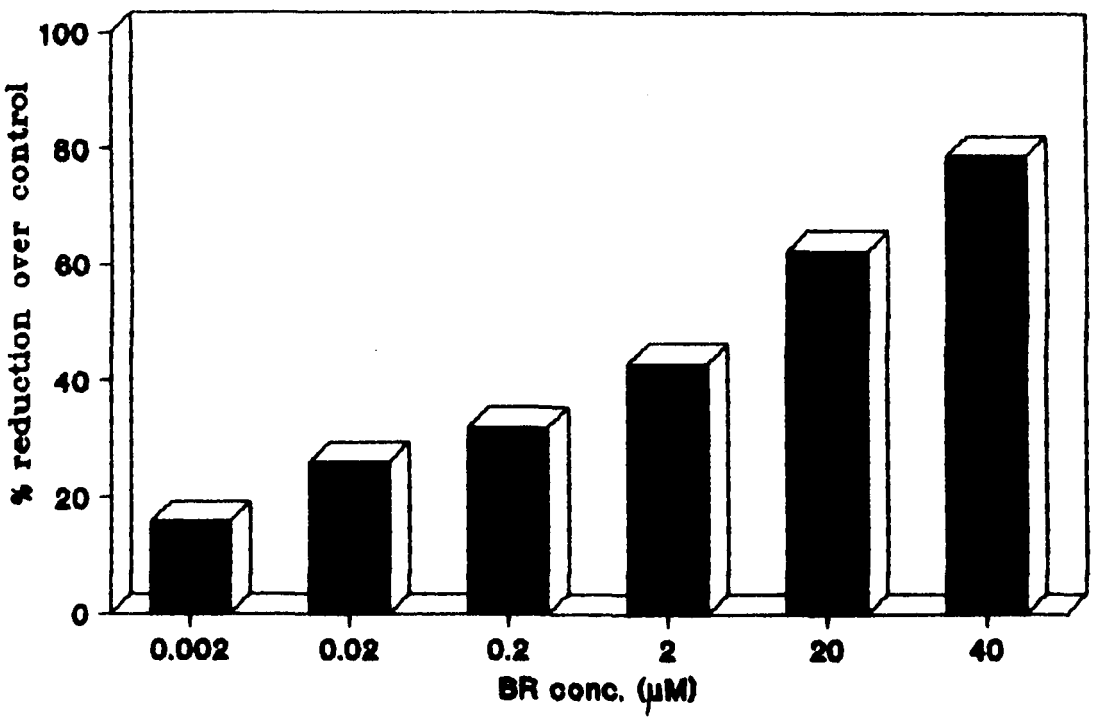


Fig. 10: Influence of different concentrations of BR on change in angle between coleus petiole.

[Coleus petiole explants were planted on agar medium. Hundred μ l of different concentrations of BR was applied to the cut end of petioles. The angle was determined at the end of 72 hours. The per cent reduction in angle (degree) over control is plotted.]

test system, a four fold reduction in curvature of angle was observed at higher concentration of BR (40 µM) used.

4.1.8 EXPANSION GROWTH OF TOBACCO LEAF DISCS

An experiment was performed to determine how excised tobacco leaf discs responds to exogenously applied BR. The BR at various concentrations ranging from 0.02 to 2 µM was evaluated in this test system. Figures 11a and 11b shows the BR effect on expansion growth and fresh weight of tobacco leaf discs. Increase in diameter of the leaf discs and increment in fresh weight of discs were recorded after incubation over the medium for a period of 20 days.

A linear increase in fresh weight as well as diameter of the leaf discs were observed with increasing concentration of BR in the medium. At a concentration of 0.2 µM BR the increase in fresh weight and diameter of the leaf discs were 57 and 10 per cent respectively over control.

4.1.9 POLLEN GERMINATION TEST IN *Leucina leucocephala*

The influence of BR on the extent of germination of pollen grains and the pollen tube length at different intervals after incubating in pollen germination medium containing BR was studied (Fig. 12a). Presence of 0.2 µM BR in the medium induced early germination, which increased with time and reached 90 per cent by 30 minutes. In the absence of BR, pollen germination was

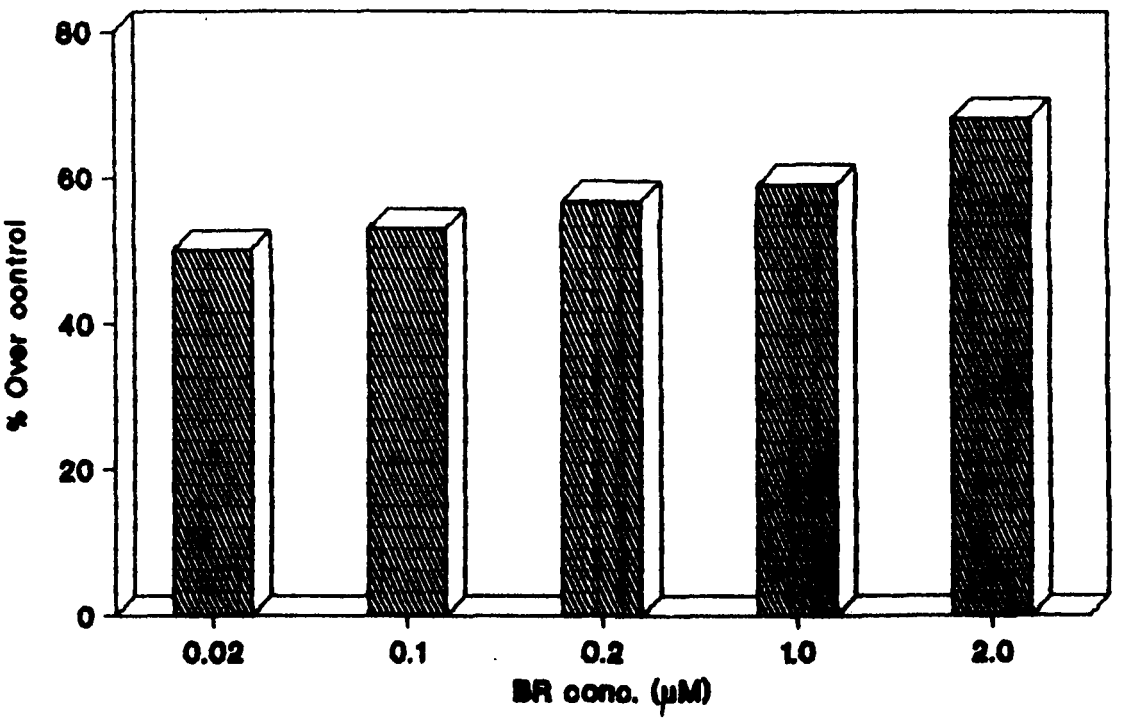


Fig. 11a: Influence of different concentrations of BR on fresh weight of tobacco leaf discs.

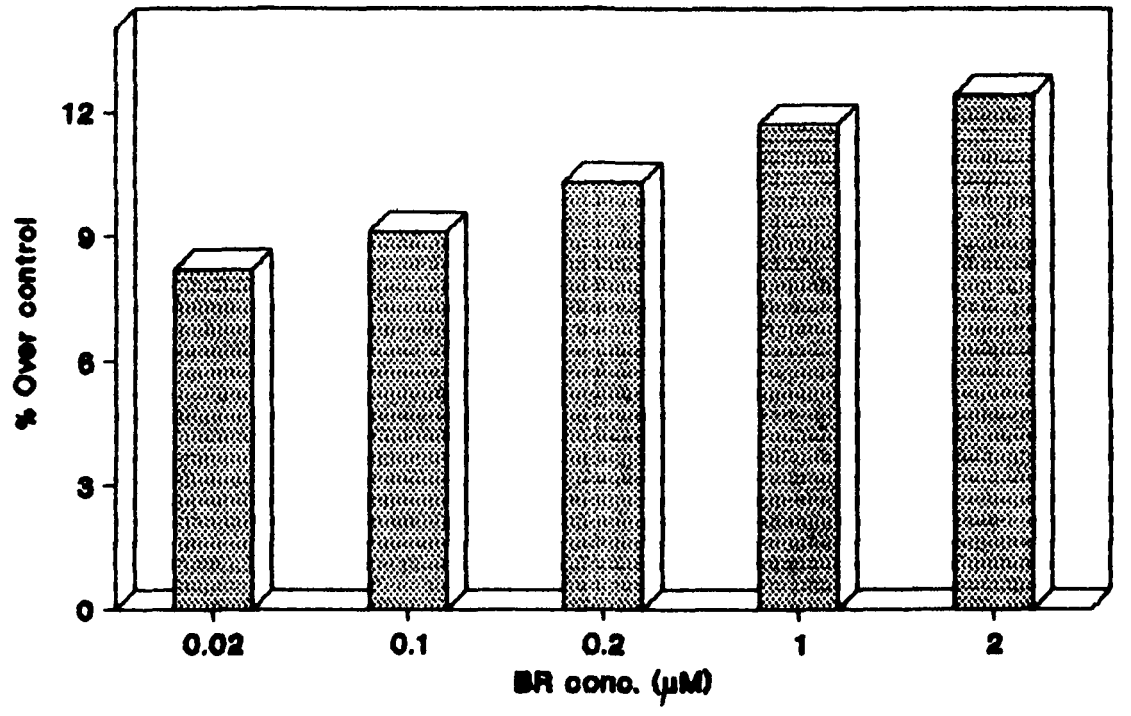
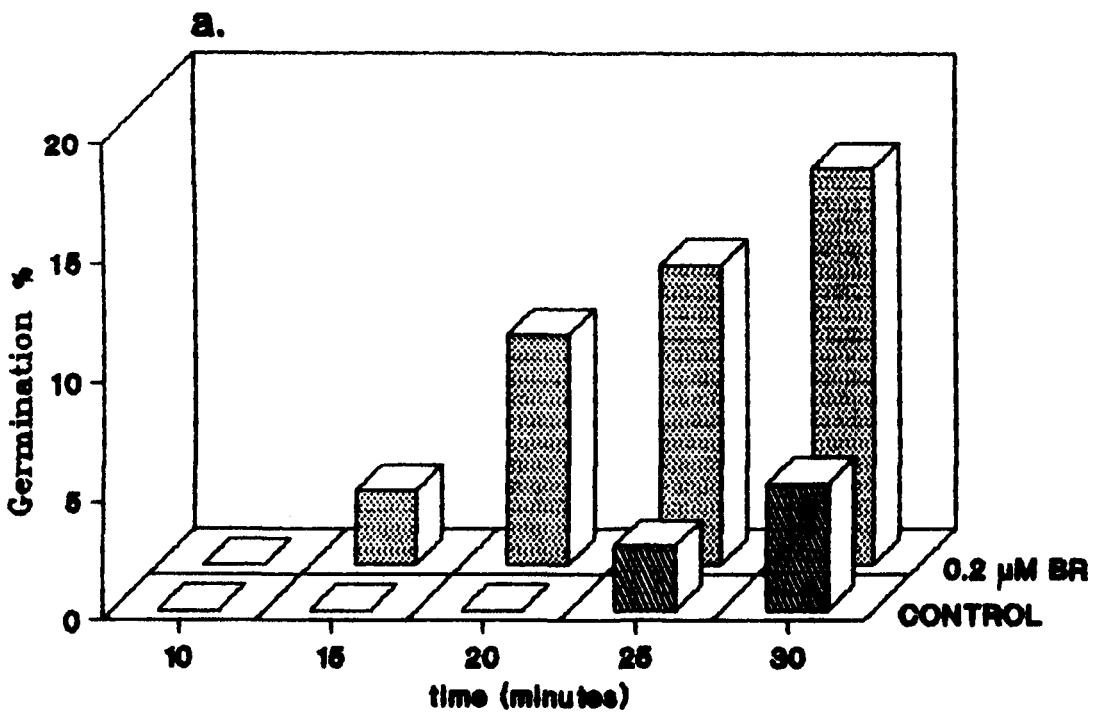


Fig. 11b: Influence of different concentrations of BR on expansion of tobacco leaf discs.

[Leaf discs of 8 mm diameter were incubated over MS medium and increase in a) fresh weight (mg.disc^{-1}), and b) leaf disc diameter (mm.disc^{-1}) were determined at the end of 20 days of incubation.]



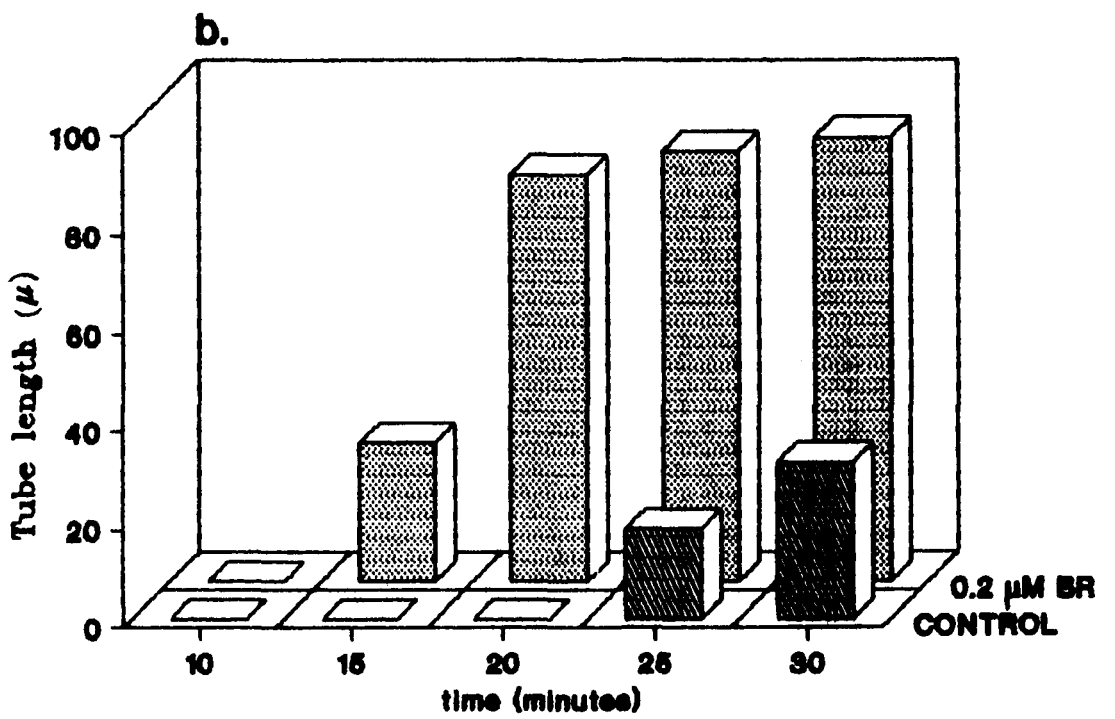


Fig. 12: Influence of BR on a) pollen germination, and b) pollen tube growth (µ) in subabul (Leucina leucocephala).

initiated only after a period of 20 minutes and at the end of 30 minutes, the germination was around 32 per cent.

Pollen tube length measured at different intervals indicated that elongation was more when BR was present in the medium. At the end of 30 minutes, pollen tube length was 17 μ compared to 5 μ over control (Fig. 12b). This observation indicate that BR induces early germination and pollen tube elongation of Leucina leucocephala.

4.1.10 RICE LEAF LAMINA INCLINATION TEST

In excised leaf segments: Rice leaf segments consisting of 1 cm long leaf lamina, a ligule and 1 cm long leaf sheath were floated on different concentrations of BR. At the end of 48 hours, the magnitude of the induced angle between lamina and sheath were measured. The effect of BR on bending of leaf segments is shown in Fig. 13a and Plate 2a.

BR dramatically induced the inclination of lamina even at low concentrations. The angle of inclination increased with increasing concentration of BR in the medium. At high concentration of BR, lamina was bent sharply at lamina joint towards the abaxial side and almost came into contact with their sheath.

In intact seedlings : In this test system of seven day old rice seedlings, 10 μl of BR or HBR was applied to the

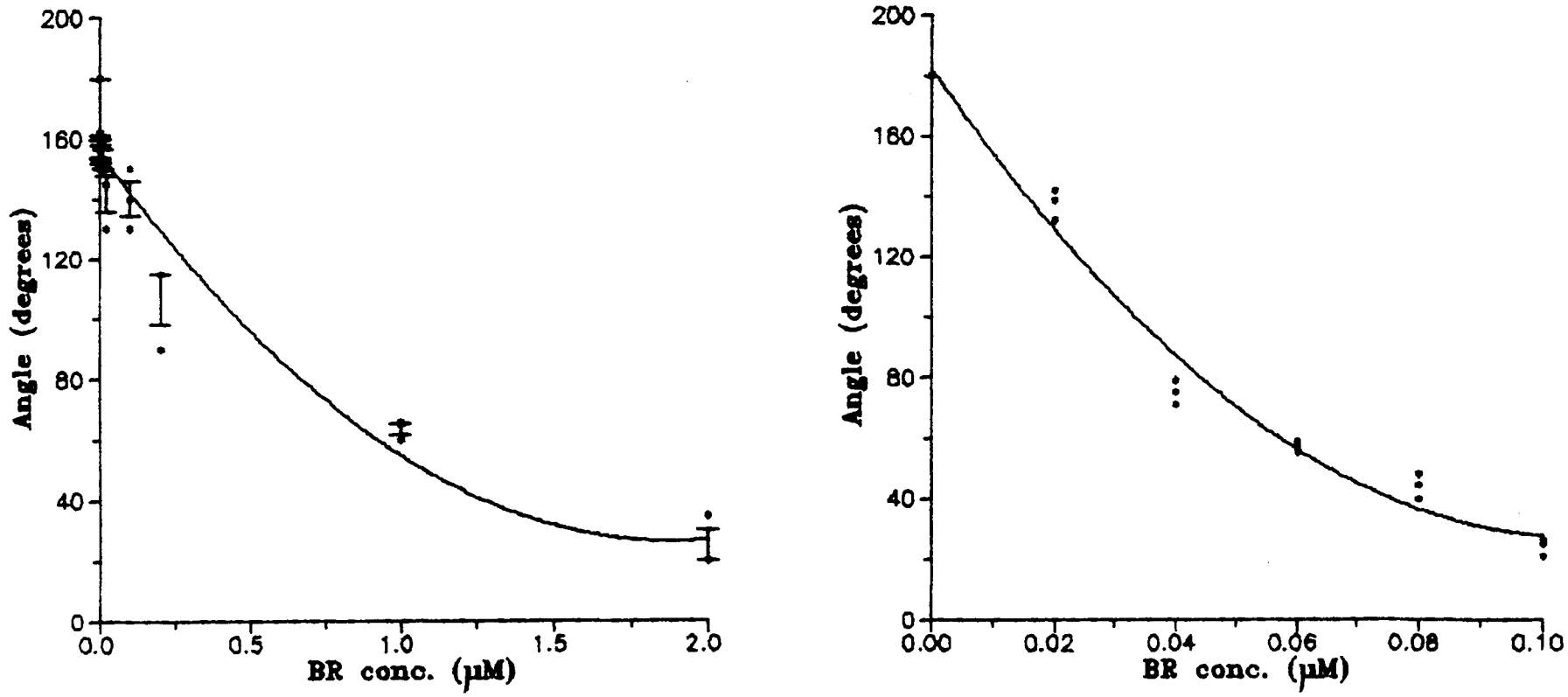


Fig.13: Influence of different concentrations of BR on rice leaf lamina inclination in a) Excised leaf segments, and b) Intact seedlings.

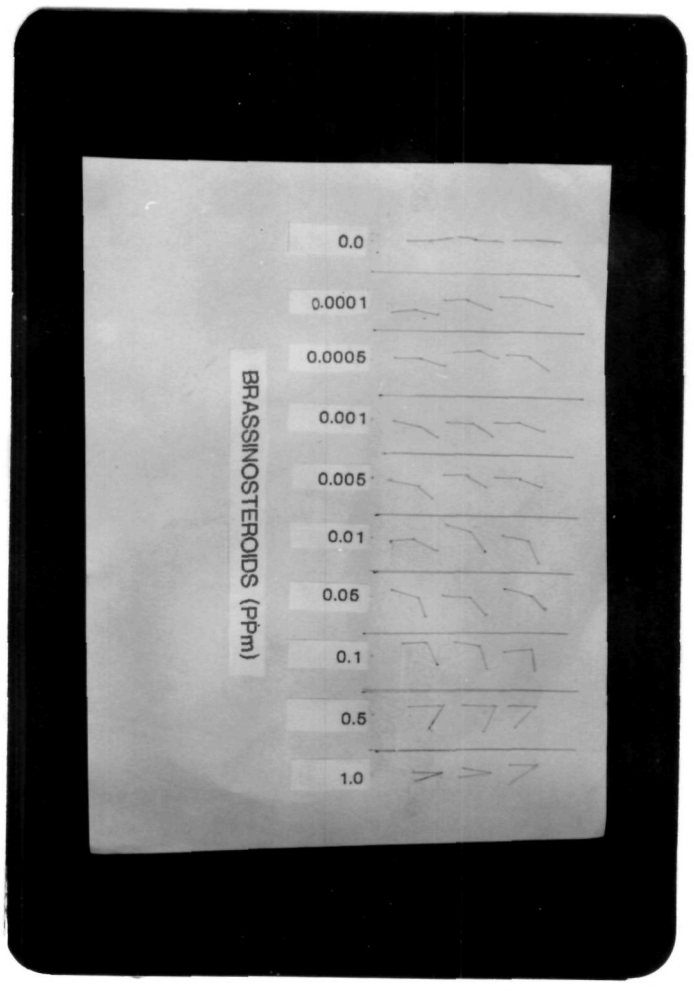


Plate 2a: Influence of different concentrations of BR on extent of leaf lamina inclination in excised rice leaf segments.

[Note: 1 ppm = 2 μM]

second leaf lamina joint (ligule) between lamina and leaf sheath. The seedlings were kept under diffused light for 48 hours and the angle of inclination between leaf sheath and lamina was measured (Fig. 13b, 13c and Plate 2b).

BR induced rice leaf lamina inclination was concentration dependent.Increase in bending was noticed in concentration range between 0.02 to 0.1 μM. Even at a concentration of 0.02 μM induced leaf lamina inclination was marked. The correlation between the induced angle of inclination and concentration indicated a linear relationship between 0.0002 to 2.0 μM concentration in excised as well as in intact test systems (Fig. 14a and 14b).

The effect of other phytohormones on rice leaf lamina inclination was examined. The hormones tested were indole acetic acid, benzyl adenine, gibberellic acid, ethylene and absicsic acid (Table. 1).

Indole-acetic acid at a concentration range between 0.0176 and 1.76 μg was ineffective in eliciting any response in this test system.

Benzyl adenine a synthetic cytokinin was used at concentration range of 0.2 to 200 ng. The results indicated that Benzyl adenine was also ineffective in inducing rice leaf lamina inclination.

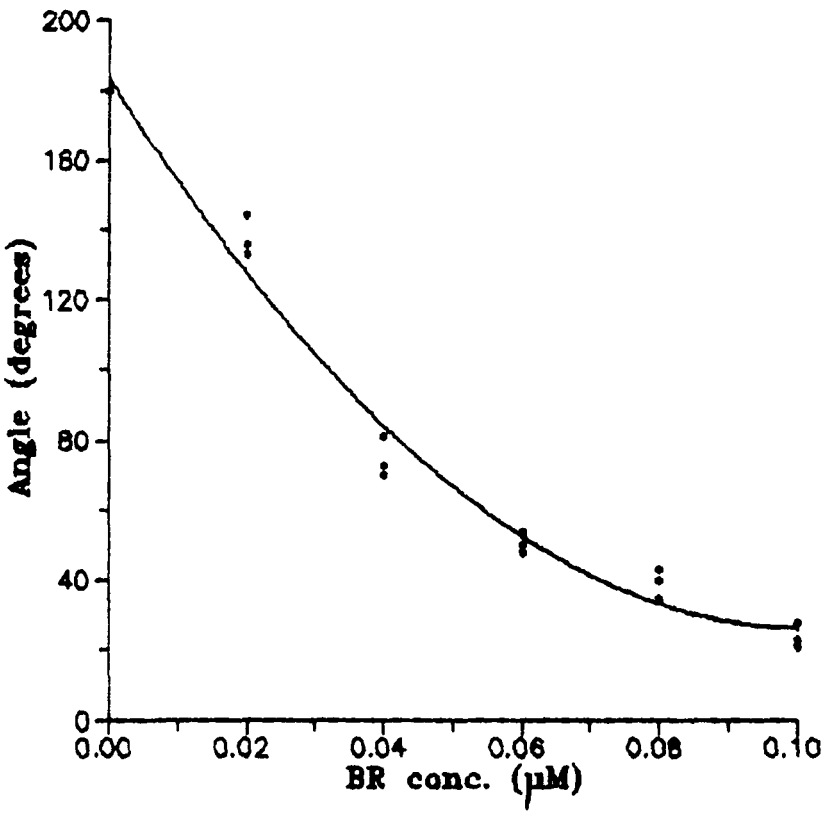


Fig. 13c : Influence of different concentrations of HBR on leaf lamina inclination in intact rice seedlings.

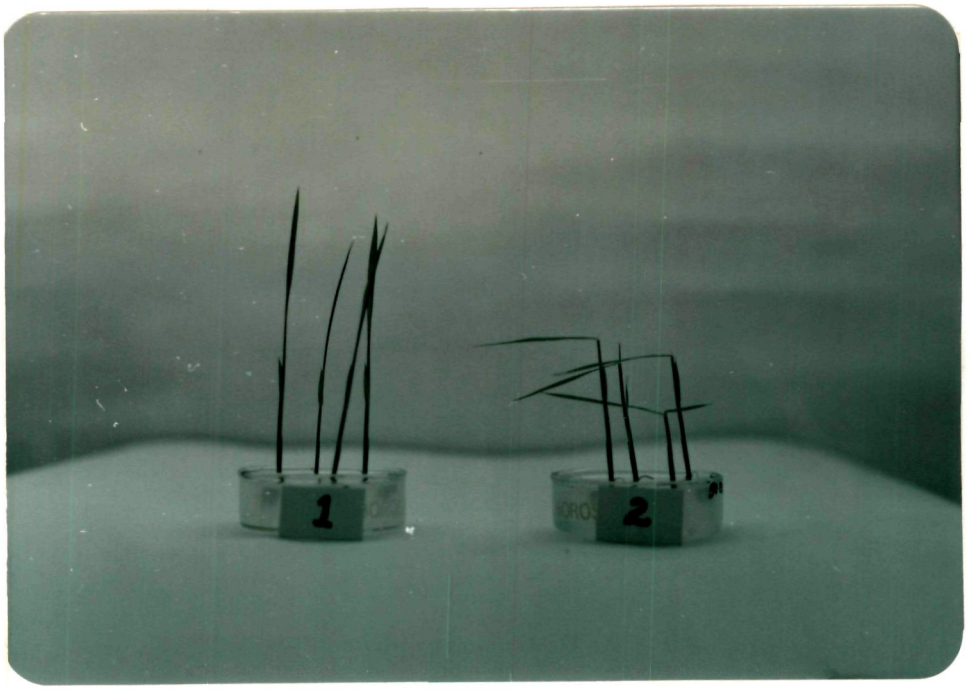
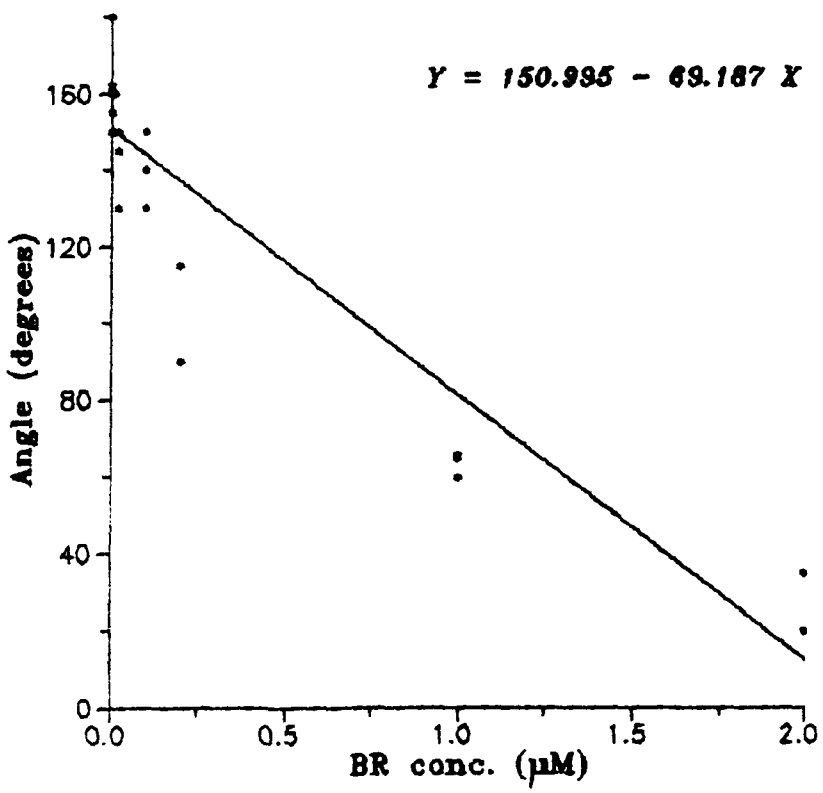


Plate 2b: Effect of BR on leaf lamina inclination in intact rice seedlings, cv. Tan-gin-bozu.

1) Control, and
2) Ø.Ø2 µM BR.

a.



b.

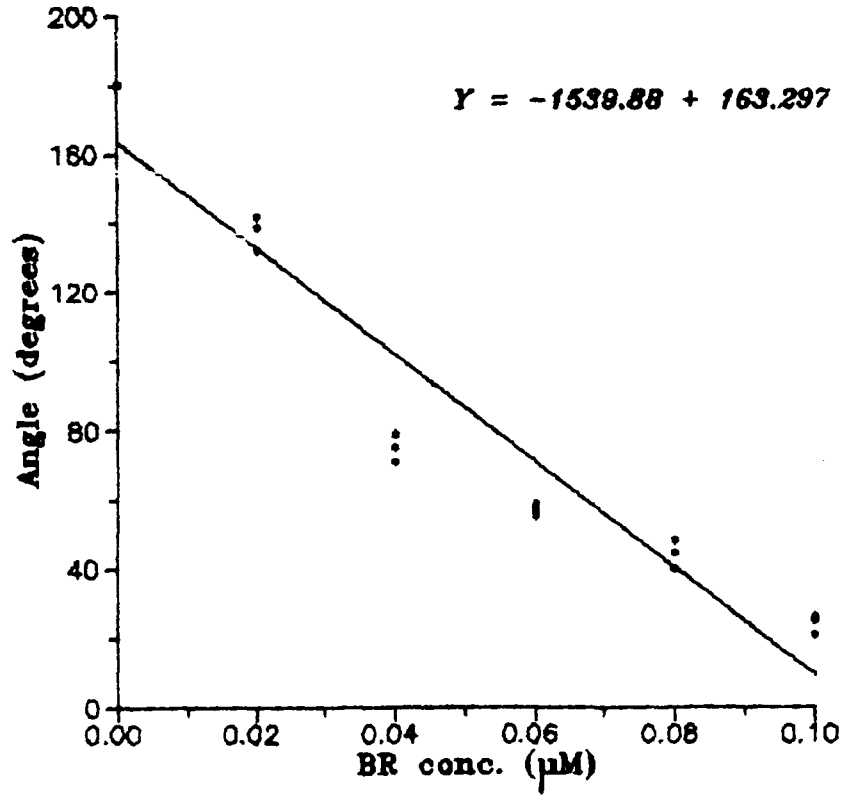


Fig. 14 : Relationship between BR concentration and extent of rice leaf lamina inclination in a) Excised leaf segments, and b)Intact seedlings.

Table 1: Influence of BR and other phytohormones on the extent of leaf lamina inclination in rice seedlings.

(Angle in degrees)

Treatments (BR µg)	0.0	0.1	0.2	0.3	0.4	0.5
Angle	180	139	79	59	33	25
Treatment (Ethephon µg)	0.0	0.01	0.1	0.25	0.5	1.0
Angle	180	180	180	180	180	180
Treatment (GA µg)	0.0	0.0342	0.171	0.342	1.71	3.42
Angle	180	180	180	180	180	180
treatment (IAA µg)	0.0	0.0176	0.088	0.176	0.88	1.76
Angle	180	180	180	180	180	180
Treatment (BA ng)	0.0	0.2	2.0	20.0	200.0	
Angle	180	180	180	180	180	
Treatment (ABA µg)	.0264	.132	.264	1.32	2.64	
Angle	180	180	180	180	180	

[Seven day old rice seedlings raised over agar medium were used. Ten µl of test solution of different concentrations of hormons were placed on the ligule of second leaf. The angle of curvature between the main axis and leaf lamina was measured at the end of 48 hours.]

Gibberellic acid at the concentration range of 0.0342 to 3.42 µg did not alter the angle between leaf lamina and leaf sheath indicating that gibberellic acid did not elicit any response in this test system.

Ethrel, a compound which releases ethylene when used in the concentration range of 0.01 to 1 µg, was also ineffective in eliciting any response in rice leaf lamina inclination bioassay.

Abscisic acid (ABA) at different concentrations ranging from 0.0264 to 2.64 µg was used. The results indicated that ABA was also not effective in inducing bending of rice leaf lamina.

These results suggest that rice leaf lamina inclination test was highly specific for brassinosteroids and other phytohormones like IAA, Cytokinins, Gibberellins, Ethylene and Abscisic acid are not effective in eliciting any response in this test system.

4.2 INTERACTION OF BR WITH AUXINS AND OTHER HORMONES IN A FEW TEST SYSTEMS

Brassinosteroids elicit a pronounced effect on stem elongation similar to that of auxins and Gibberellins in many test systems. Induced stimulation of Brassinosteroids in growth has been found in segments of wheat coleoptile, maize mesocotyl, mungbean hypocotyl, and soybean stem.

In this part of the results, the interaction effect of BR with auxins on growth responses were tested in a few systems, namely Wheat coleoptile segment, rice leaf lamina and tobacco leaf disc. In addition to the influence of auxins, cytokinin interaction with BR was studied in expansion growth of excised cucumber cotyledons.

4.2.1 INTERACTION OF BR WITH IAA IN THE ELONGATION OF WHEAT COLEOPTILE SEGMENTS

The interaction effect of BR with IAA on elongation growth of wheat coleoptile segments was studied by incubating the coleoptile segments in media containing varying concentrations of IAA and BR.

When IAA concentration was kept constant and BR concentrations were varied, final length of the segments increased with increasing concentrations of BR (Fig. 15). The final length of coleoptile was more at 0.02 μM BR and 10 μM concentration of IAA. This suggests that in presence of both BR and IAA, the elongation growth was more than that of either BR or IAA alone,indicating that the interaction effect was additive.

This set of data are indicative of marked interaction effect between IAA and BR in eliciting higher growth rate of coleoptile segments.

4.2.1.1 INFLUENCE OF SEQUENTIAL TREATMENT OF BR AND IAA ON ELONGATION GROWTH OF COLEOPTILE SEGMENTS

The influence of sequential treatments of IAA and BR on coleoptile growth was studied to know further about

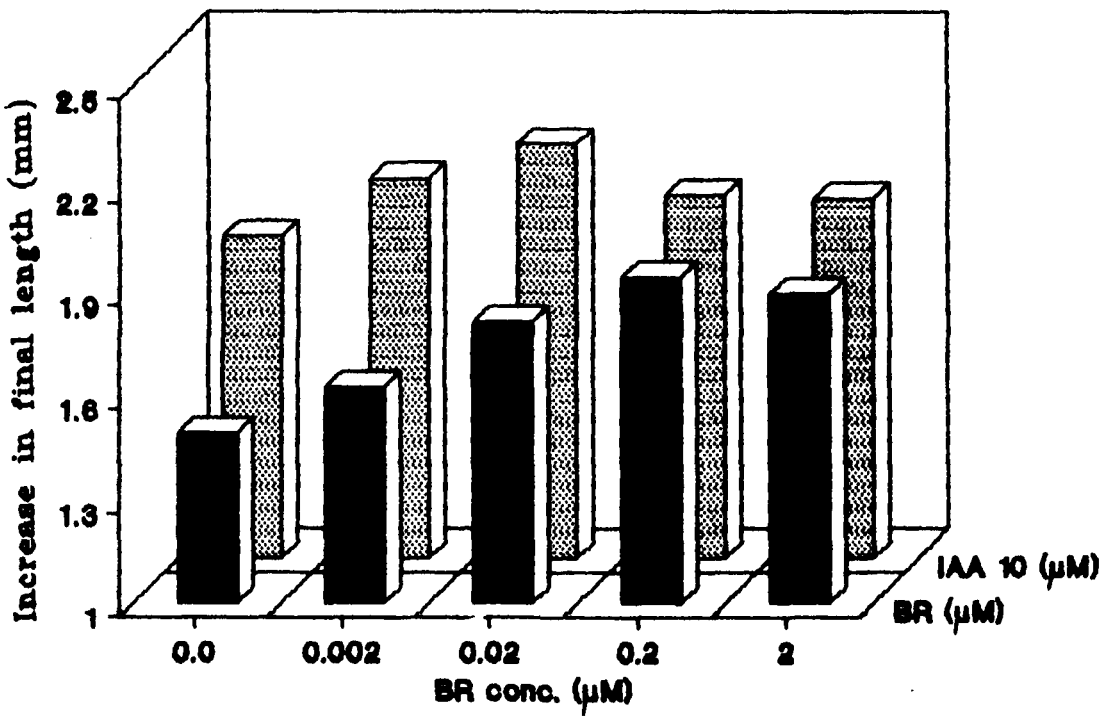


Fig. 15: Influence of different concentrations of BR with IAA on elongation of wheat coleoptile segments.

the interaction effect of IAA and BR. Coleoptile segments were pretreated in 0.2 μM BR for 1 to 4 hours duration and then transferred to IAA (10 μM) medium for post treatment for a period of 24 hours (Table 2a). BR pretreatment enhanced the IAA induced growth, and maximum elongation was observed when the BR pretreatment was given for a period of 3 hours. Increasing the pretreatment duration beyond 3 hours had no significant effect on final length of the coleoptile segments compared to 3 hour pretreatment.

Keeping the optimum duration of pretreatment as 3 hours, the influence of pretreatment on growth was studied in a range of concentrations of BR from 0.02 to 20 μM. Synergistic effect of BR and IAA (10 μM) was observed at 0.2 μM BR, and coleoptile segment length increased with increasing concentration of BR upto 2.0 μM (Table 2b). The extent of synergistic effect did not increase further when BR concentration was beyond 2.0 μM.

In another set of experiment, the sequence of treatment was reversed by giving IAA treatment for 3 hours followed by BR post treatment for a duration of 24 hours. IAA pretreatment did not influence the BR induced elongation markedly (Table 2c).

To study whether the pretreatment effect of BR is stable, the coleoptile pretreated in BR was floated over

Table 2a : Effect of BR pretreatment on IAA induced elongation of wheat coleoptile segments.

Pretreatment	Post treatment(24hr)	Final length(mm)
Water	Water	14.6 ± 0.10
IAA 10 µM (4 hrs)	IAA 10 µM	19.6 ± 0.13
BR (0.2 µM)		
1 hour	IAA 10 µM	20.3 ± 0.13
2 hours	"	23.2 ± 0.13
3 hours	"	23.8 ± 0.15
4 hours	"	23.7 ± 0.14
CD at 5%		1.2 ± 0.06

[Coleoptile segments were pretreated with BR (0.2 µM) for different durations and then transferred to IAA (10 µM) medium for a further period of 24 hours. The final length of coleoptile segments were measured after post treatment of IAA.]

Table 2b : Effect of different concentrations of BR pretreatment on IAA induced elongation of wheat coleoptil segments.

Pretreatment (3 hrs)	Post treatment (24 hrs)	Final length (mm)
Water	Water	14.6 ± 0.10
IAA 10 µM	IAA 10 µM	19.7 ± 0.12
Water	"	19.5 ± 0.11
BR 0.02 µM	"	19.7 ± 0.12
" 0.2 µM	"	23.8 ± 0.14
" 2.0 µM	"	24.1 ± 0.14
" 20.0 µM	"	24.0 ± 0.14
CD at 5%		2.6 ± 0.04

[Coleoptile segments were pretreated for 3 hours with different concentrations of BR and then transferred to IAA (10 µM). After 24 hours of incubation in IAA final length of coleoptile segments were measured.]

Table 2c : Effect of IAA pretreatment on BR induced elongation of wheat coleoptile segments.

Pretreatment (3hr)	Post treatment (24hr)	Final length (mm)
Water	Water	14.5 ± 0.10
BR 0.2 µM	BR 0.2 µM	19.0 ± 0.09
Water	"	19.0 ± 0.09
IAA 1 µM	"	20.5 ± 0.10
IAA 10 µM	"	20.8 ± 0.11
CD at 5%		2.0 ± 0.04

[Coleoptile segments were pretreated for 3 hours with different concentrations of IAA and then transferred to BR (0.2 µM). after 24 hours of incubation in BR final length of coleoptile segments were measured)

Table 2d : Effect of washing after BR pretreatment on IAA induced elongation of wheat coleoptile segments.

Pretreatment	Post treatment (24hr)	Final length (mm)
Water (3hr)	Water	14.5 ± 0.09
Water (3hr)	IAA 10 µM	19.5 ± 0.11
BR 0.2 µM (3hr)	"	23.5 ± 0.12
BR 0.2 µM (3hr) followed by floating on water for 2 hrs.	"	20.6 ± 0.12
CD at 5 %		1.3 ± 0.04

distilled water for a duration of two hours and then transferred to IAA medium for a further period of 24 hours. Floating the coleoptile segments over water after pretreatment in BR significantly reduced the pretreatment effect of BR (Table 2d).

4.2.2 INTERACTION OF BR WITH IAA IN THE RICE LEAF LAMINA INCLINATION TEST

Experiments conducted with BR indicated that, this chemical is effective in modifying leaf lamina inclination in rice seedlings. None of the other phytohormones were effective in modifying lamina inclination in rice seedlings. In many other test systems IAA has been reported to be synergistic with BR in eliciting growth responses. With an objective to study the interaction effect of BR and IAA, an experiment was conducted in this test system.

BR at a very low concentration of 0.02 µM induced lamina bending and the angle of bending was 110 degree compared to 180 degree in control. IAA alone between the concentration range of 0.01 to 1 mM was not effective in inducing lamina bending. However, combination treatment of BR+IAA was effective in inducing lamina inclination more than that of BR alone (Fig. 16 and Plate 3). At a concentration of 0.02 µM of BR along with varying concentrations of IAA, the extent of bending increased with increasing concentrations of IAA. For

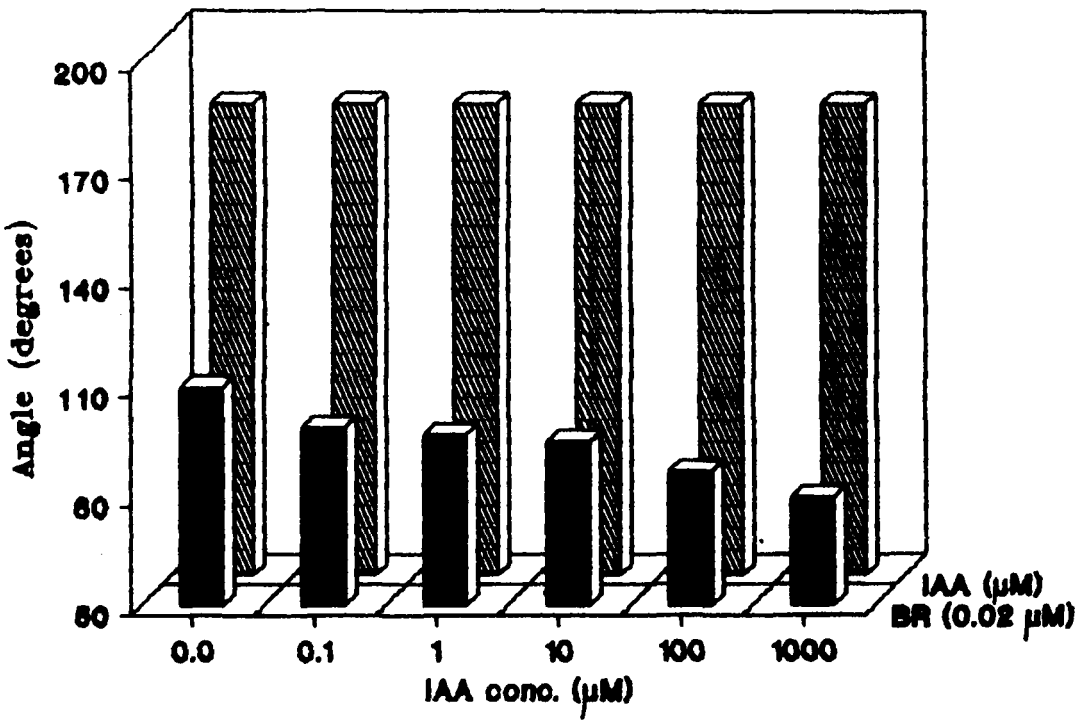


Fig. 16: Influence of different concentrations of IAA with BR on extent of leaf lamina inclination in rice seedlings.

[Known concentration of BR solutions were mixed with different concentrations of IAA. The mixture of BR+IAA solution (1Ø μl) was applied to the ligule of the second leaf of rice seedlings. Angle of inclination was measured 48 hours after application of test solutions.]

1 2 3 4

Plate 3: Interaction effect of BR with IAA on rice leaf lamina inclination: 1) Control, 2) IAA 1Ø μM, 3) BR Ø.Ø2 μM and 4) BR + IAA.

Plate 4: Interaction effect of BR with NAA on leaf expansion of excised tobacco leaf discs.

[Note: 1 ppm BR = 2 μM]

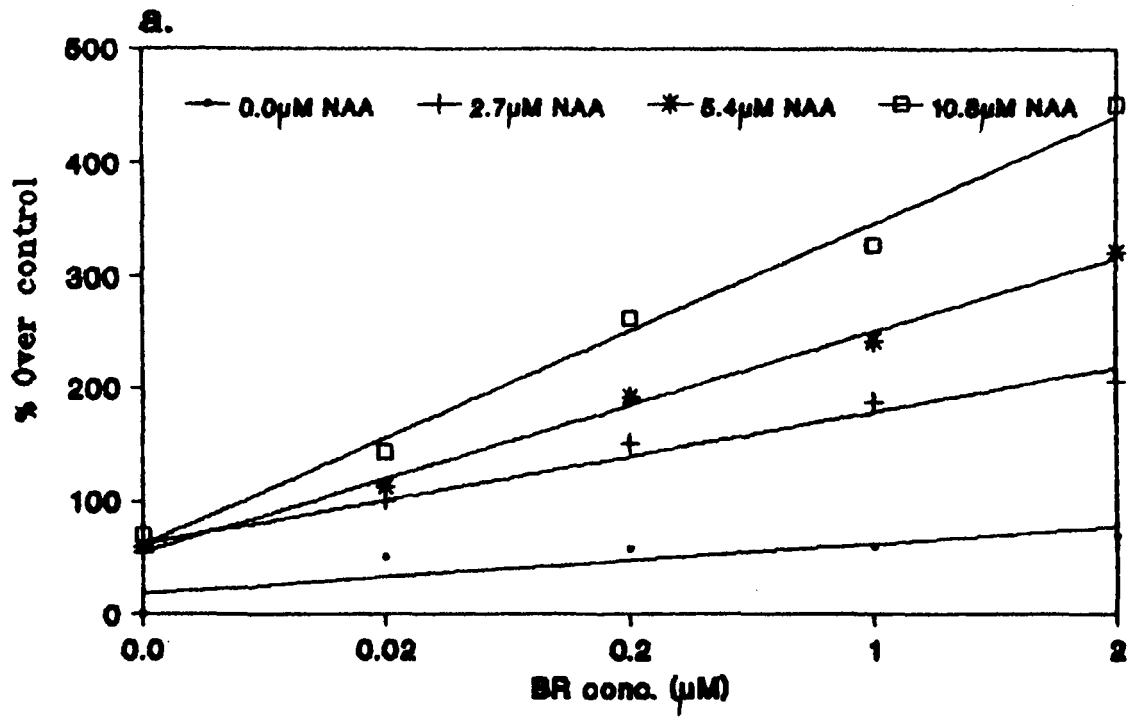
example, the angle of bending was 88 degrees at 10 uM + 0.02 μM BR compared to 110 degrees in 0.02 μM BR alone.

4.2.3 INTERACTION OF AUXIN (NAA) WITH BR IN TOBACCO LEAF DISC EXPANSION TEST

The interaction effect of BR and NAA on expansion of tobacco leaf discs was tested . Tobacco leaf discs were planted on MS agar medium with different concentrations of BR or NAA or in combination.

Fresh weight of the leaf discs measured 20 days after placing on agar medium indicated that with increasing concentration of BR in the medium, there was an increase in fresh weight of the leaf discs (Fig. 17a). This trend was noticed in the absence of NAA in the medium and also with different concentrations of NAA. BR was highly effective in increasing fresh weight at high concentrations of NAA in the medium . For example when the medium was devoid of NAA, the increase in fresh weight with 2 μM concentration of BR was 68 per cent of control. The fresh weight increase in 5.4 and 10.8 μM NAA with 2 μM BR was 380 and 450 per cent, respectively.

Leaf expansion rate measured in terms of increase in leaf disc diameter showed a similar trend (Fig. 17b and Plate 4). When 2 μM BR was present in the medium, the per cent increase over control in leaf disc diameter at 0, 5.4 and 10.8 μM NAA were 12, 52 and 101 per cent respectively. These results clearly indicate that BR and



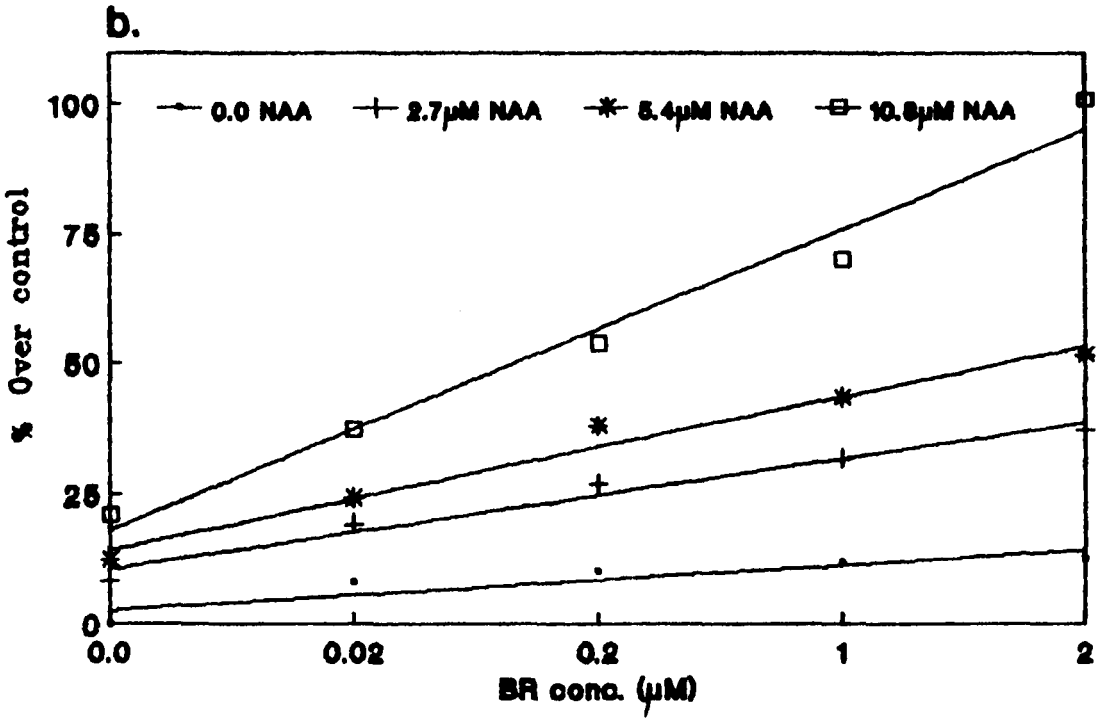


Fig. 17: Interaction effect of different concentrations of BR with NAA on a) fresh weight, and b) expansion of tobacco leaf disc.

auxins act synergistically in increasing cell expansion as well as fresh weight of tobacco leaf discs.

4.2.4 INTERACTION OF BR AND CYTOKININS IN CUCUMBER COTYLEDON EXPANSION BIOASSAY

Cytokinins were shown to be highly effective in inducing expansion of cucumber cotyledons. Experiments conducted with BR indicated that, this chemical was not effective in inducing cotyledon expansion. The effect of BR with cytokinin with and without potassium chloride in the incubation medium was tested to study the interaction effect, if any, between BA and BR in inducing cotyledon expansion.

Isolated cucumber cotyledons were incubated in medium containing different concentrations of BR, BR+BA and BR+BA+KCl. A marginal increase in weight of the cotyledon was observed with increasing concentration of BR alone. In presence of BA 6.5 μM, increasing concentrations of BR, increased the fresh weight of cotyledons to an extent of 11 to 42 per cent of BA alone. In presence of BA + KCl significant increase in fresh weight of the cotyledon over BA alone was observed. However, further increase in fresh weight due to the presence of different concentrations of BR was marginal. These results indicate that there was a marginal increase in fresh weight of the cotyledons in the presence of BR and BA together in the medium (Table 3).

Table 3 : Interation of BR with cytokinins (BA) on fresh weight of cucumber cotyledons.

(Fresh weight mg. cotyledon^{-1})

Treatments (BR conc.)	Water	BA (6.5 µM)	BA (6.5 µM)+KCl(75mm)
0.00	7.71 ± 0.41	18.23 ± 0.89	31.00 ± 1.70
0.002 µM	7.85 ± 0.41	20.21 ± 0.90	31.49 ± 1.81
0.02 µM	7.98 ± 0.43	21.37 ± 0.86	33.52 ± 1.80
0.2 µM	8.02 ± 0.46	24.32 ± 0.96	33.40 ± 1.74
2.0 µM	8.20 ± 0.43	25.48 ± 1.02	33.46 ± 1.20
20.0 µM	8.59 ± 0.46	25.91 ± 1.12	33.54 ± 1.31
CD at 5 %	2.10 ± 0.26		

4.3 COMPARISON OF IAA AND BR INDUCED ELONGATION GROWTH OF COLEOPTILE SEGMENTS

The activity of BR was compared with that of IAA at various durations after the incubation of coleoptile segments in water, BR and IAA. The effect of these treatments on elongation was determined by measuring length of coleoptile segments at 10 minutes interval up to 120 minutes and then at 30 minutes intervals upto 240 minutes. Marked variation between the treatments was observed after incubation up to 120 minutes. When coleoptile segments were incubated in IAA, the length increased significantly after 30 minutes incubation. A linear increase in length was observed from 30 minutes to 120 minutes in IAA and at the end of 120 minutes, the length was 35 and 36 per cent more than that of coleoptile incubated in water and BR, respectively (Fig. 18a). The elongation of coleoptile segments were more in BR compared to water. However, at the end of 120 minutes the difference in length of coleoptile in BR medium was only 5 per cent more than that of control.

The length of coleoptile measured at hourly intervals upto 4 hours and at 3 hours interval thereafter, indicated significant difference in length in BR and IAA treatments (Fig. 18b). A linear increase in growth was observed in IAA and BR from 1 to 13 hours. The extent of increase in length was less between 13 hours and 24

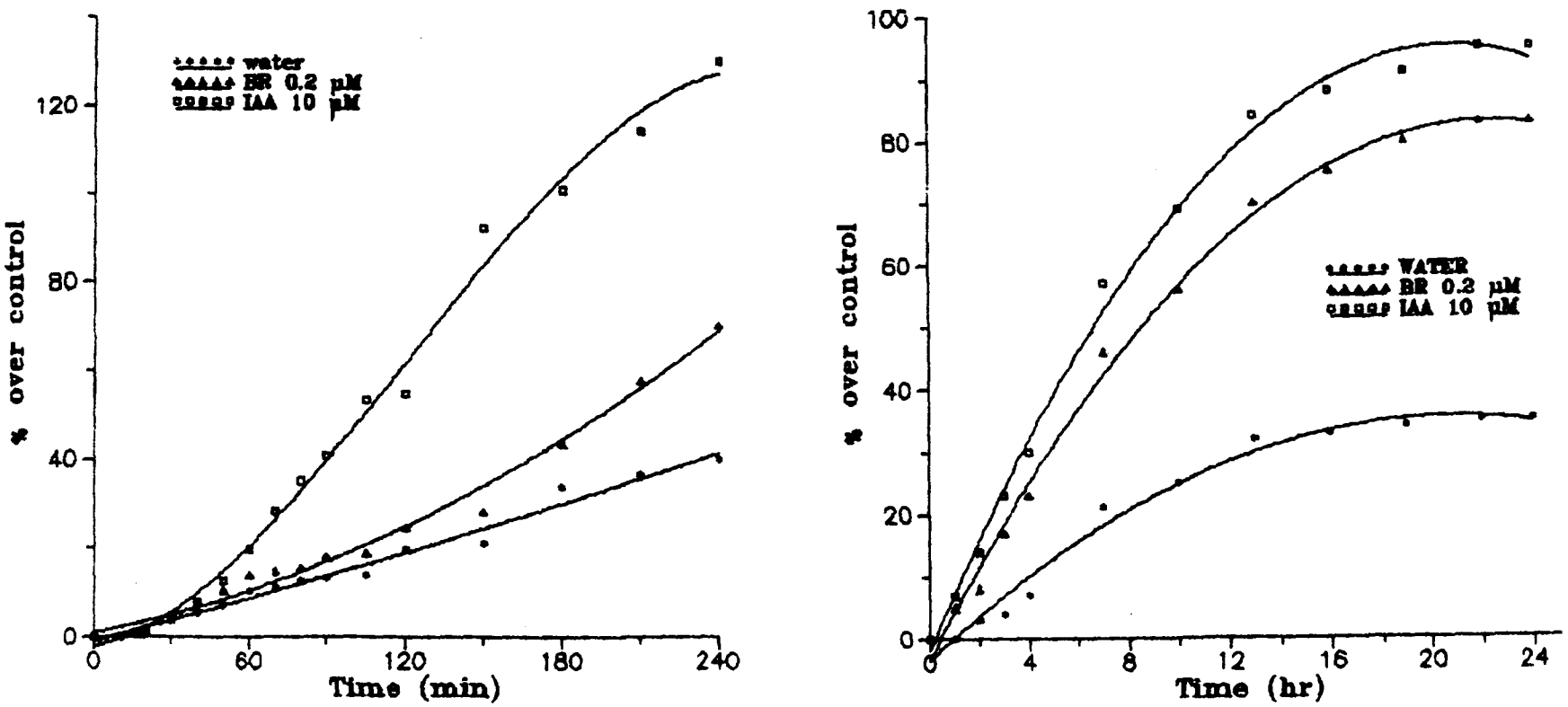


Fig. 18 : Cumulative per cent increase in length of coleoptile segments at different intervals after floating over water, IAA and BR medium.

hours. Although, the elongation of coleoptile in water medium was also maximum between 1 and 12 hours, the increase was less compared to BR and IAA.

IAA treatment resulted in increased growth of coleoptiles from 30 minutes after start of the experiment. Initially, the elongation was more in IAA medium upto 130 minutes. During this period the increased growth in water and in BR treatment was relatively less. Appreciable increase in growth of coleoptile segments was observed only from 2 hours after incubation in the BR medium.

4.3.1 EFFECT OF IAA AND BR ON ACIDIFICATION OF INCUBATION MEDIA

Auxins induced rapid elongation response in coleoptile and stem segments were shown to be due to induced acidification of cell wall. Auxin induces proton movement from cytoplasm to cell wall, resulting in acidification of cell wall, which leads to cell wall loosening and cell growth. During this process the pH of the medium also reduces (Fig. 19).

The effect of BR on acidification of medium was studied and compared with that of auxin induced acidification. Coleoptile segments were incubated in water, IAA (10 uM), BR (0.2 uM), and IAA + BR media. When coleoptile segments were incubated in IAA medium, a reduction of pH of the medium was observed after 1 hour

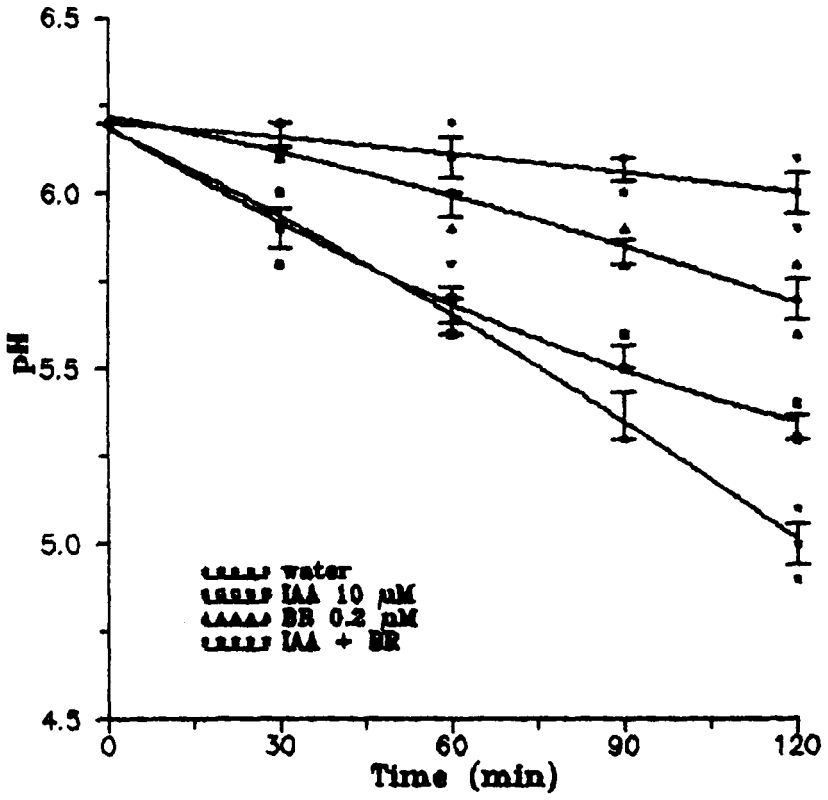


Fig. 19: Influence of IAA, BR and IAA + BR on acidification of incubation media.

incubation. Appreciable reduction in pH in BR medium was observed at the end of 90 minutes of incubation. When both IAA and BR were in the medium, acidification of medium was achieved at 30 minutes of incubation.

The relationship between pH of the medium and increase in growth at different intervals after incubation was worked out from the pooled data, as shown in Fig. 20. A negative and significant relationship was observed between reduction in pH of the medium and increase in length of the coleoptile segments.

4.3.2 EFFECT OF INHIBITORS ON IAA AND BR INDUCED GROWTH OF WHEAT COLEOPTILE SEGMENTS

In order to find out essential differences in the mode of action of BR and IAA, some experiments were done by using the inhibitors like galactose which inhibits the formation of UDP-glucose and the incorporation of glucose into the cell wall, dicyclohexylcarbodiimide (DCCD), which inhibits the membrane bound ATPase, potassium cyanide (KCN), a metabolic inhibitor, which inhibits oxidative phosphorylation, and protein synthesis inhibitors like cycloheximide (CH), 5-fluorouracil (5-FLU),and chloramphenicol.

4.3.2.1 EFFECT OF GALACTOSE ON IAA AND BR INDUCED COLEOPTILE GROWTH

Galactose inhibits auxin induced growth of avena coleoptile segments by inhibiting cell wall synthesis

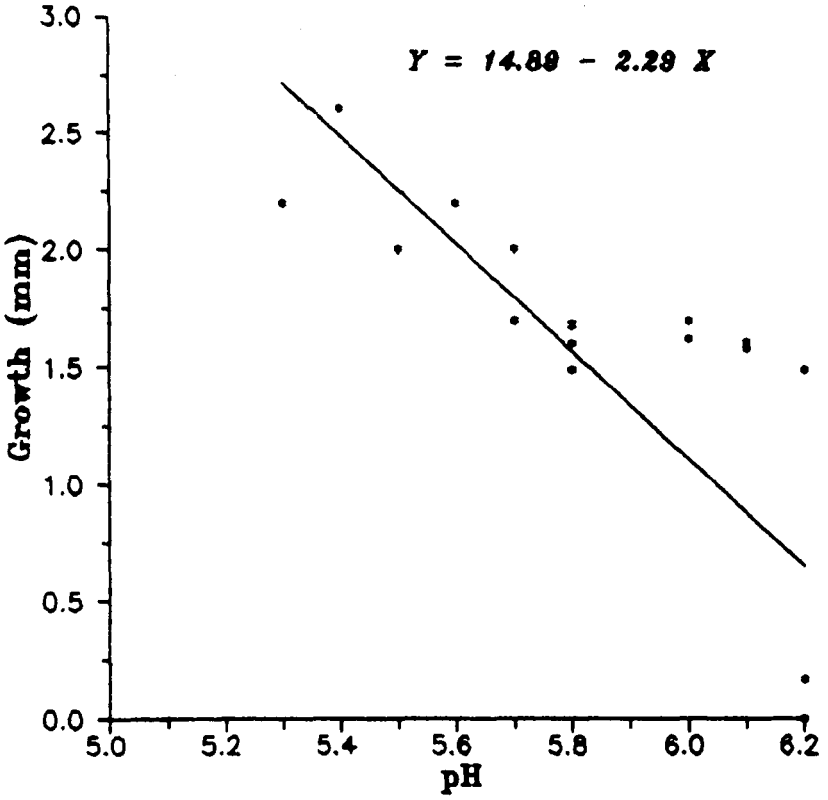


Fig. 20: Relationship between IAA and BR induced growth and acidification of incubation media.

(Cheung and Cleland, 1991). This experiment was conducted with an objective to know whether BR induced elongation growth is also mediated by increased stimulation of cell wall synthesis. Wheat coleoptile segments were incubated in IAA (10 μM) and BR (0.2 μM) with and without different concentrations of galactose. The final length of coleoptile segments were recorded at the end of 24 hours of incubation period as shown in Fig. 21a. A concentration dependent reduction in coleoptile growth was found with increase in concentration of galactose in the medium. At 10 mM concentration of galactose there was a marked reduction in growth in basal, IAA, as well as in BR media. At a 50 mM concentration of galactose growth of coleoptile in basal, IAA and BR media were inhibited to an extent of 47, 62 and 54 per cent, respectively.

Coleoptile segments were pretreated with 10 mM galactose and then transferred to IAA and BR media. The galactose was effective in inhibiting both IAA and BR induced growth but the effect on BR induced growth was relatively less (Fig. 21b). This indicates that galactose was effective in inhibiting BR induced growth only, when, it was present along with BR throughout the incubation period.

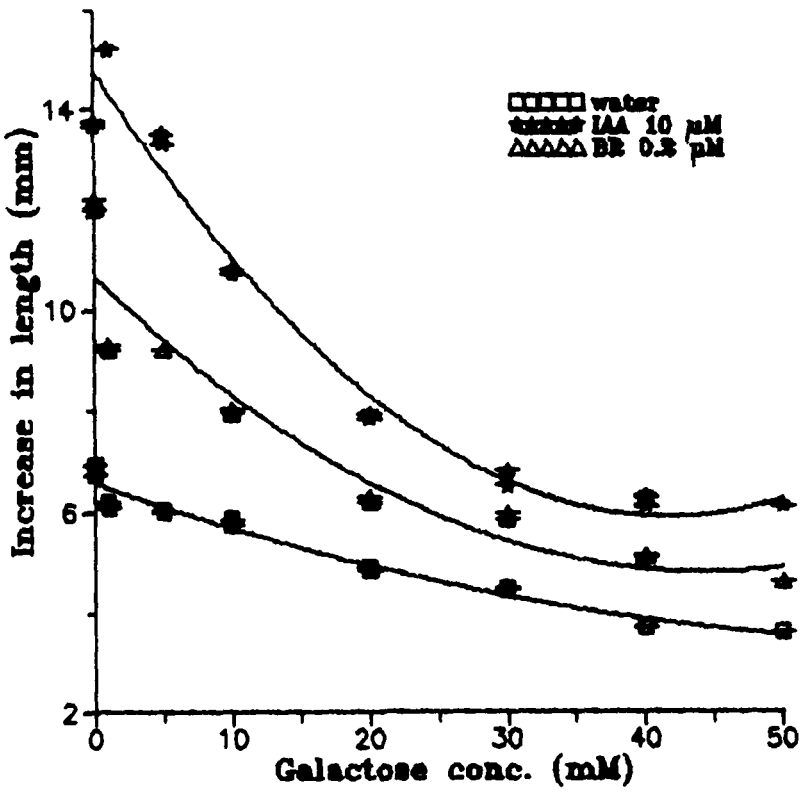


Fig. 21a: Effect of different concentrations of galactose on IAA and BR induced elongation in wheat coleoptile.

(Coleoptile segments were incubated in medium containing BR (Ø.2 μ M) or IAA (1Ø μ M) with different concentrations of galactose. Length of coleoptile segments were measured 24 hours after incubation)

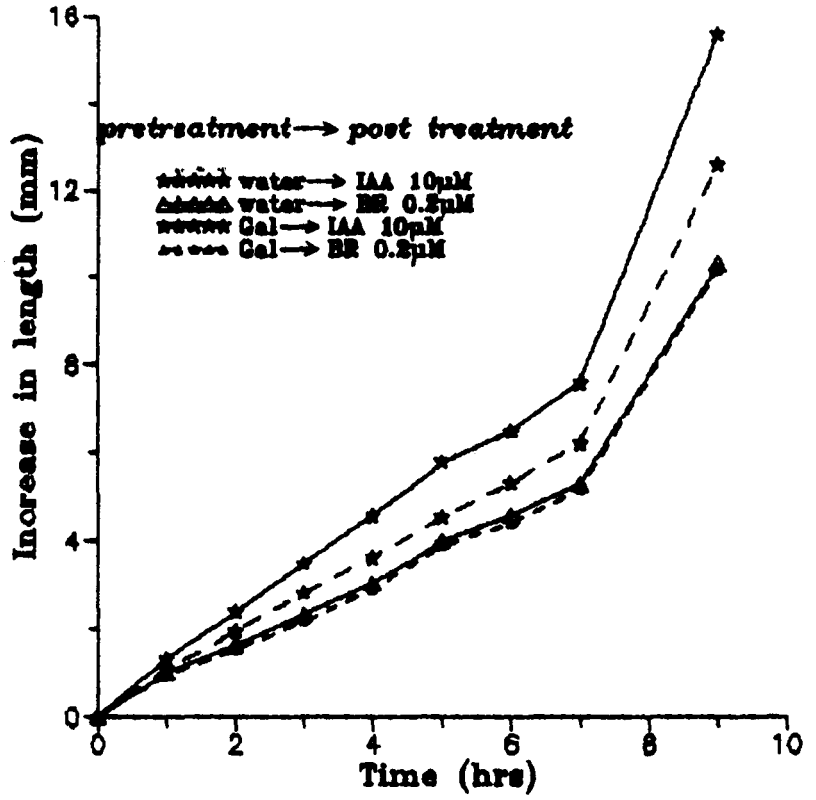


Fig. 21b: Pretreatment effect of galactose on BR and IAA induced growth in wheat coleoptile segments.

[Coleoptile segments were pretreated with Ø or 1Ø mM galactose for 9Ø minutes and then transferred to IAA (1Ø μM) or BR (Ø.2 μM). Coleoptile length were measured at different intervals of post treatment]

4.3.2.2 EFFECT OF METABOLIC INHIBITORS ON IAA AND BR INDUCED GROWTH OF COLEOPTILE SEGMENTS

Potassium cyanide (KCN), a metabolic inhibitor, which inhibits oxidative phosphorylation was used to study its effect on IAA and BR induced growth. When KCN was present in the medium with IAA and BR, significant reduction in growth was observed above a concentration of 500 μM KCN (Fig. 22).

The influence of a membrane bound ATPase inhibitor dicyclohexylcarbodiimide (DCCD) was tested on IAA and BR induced elongation of coleoptile segments. DCCD was effective in inhibiting the growth of coleoptile segments in basal, IAA and BR media. The DCCD induced inhibition of growth increased with increase in concentration of DCCD. At a concentration of 100 μM DCCD, the length of coleoptiles were almost similar in basal, IAA and BR media (Fig. 23a).

The time course of elongation growth in presence of different concentrations of DCCD along with IAA and BR were studied, to know whether there is any lag phase in DCCD induced inhibition. At a concentration of 10 μM DCCD was effective in inhibiting IAA induced growth after 10 minutes of addition of DCCD into the medium and the effect of DCCD in inhibiting IAA was visible after 30 minutes after addition of DCCD (Fig. 23b). BR induced increase in elongation growth was observed only at 90

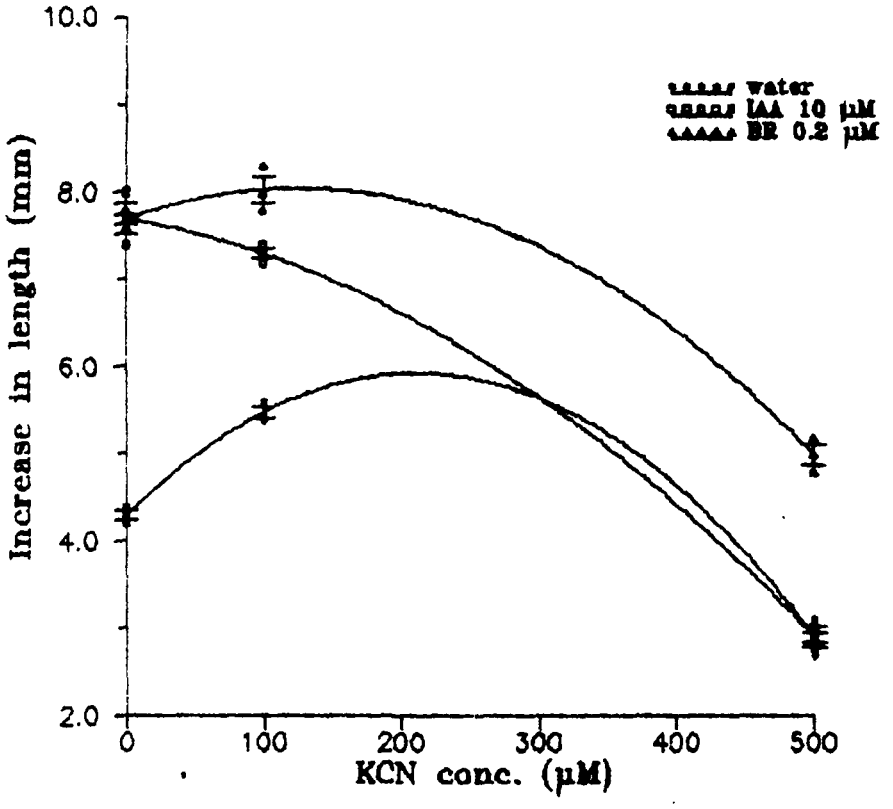


Fig. 22: Effect of different concentrations of KCN on IAA and BR induced growth in wheat coleoptile segments.

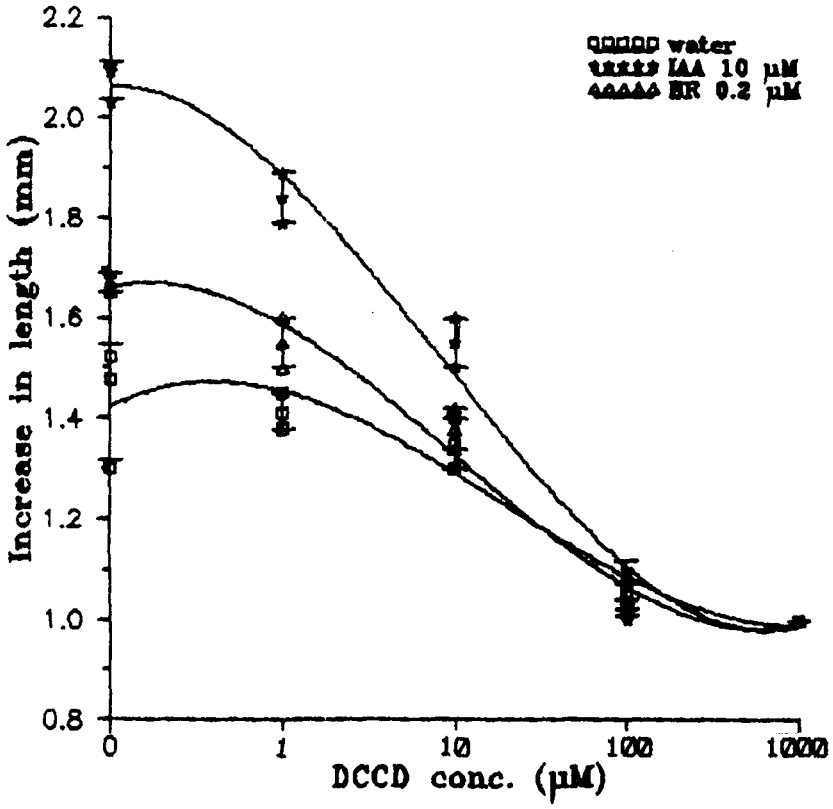


Fig. 23a: Effect of different concentrations of DCCD on BR and IAA induced growth in wheat coleoptile.

[Coleoptile segments were incubated in water IAA (10 µM) or BR (0.2 µM) with different concentrations of DCCD. The final length of coleoptile segments were measured at the end of 24 hours incubation period]

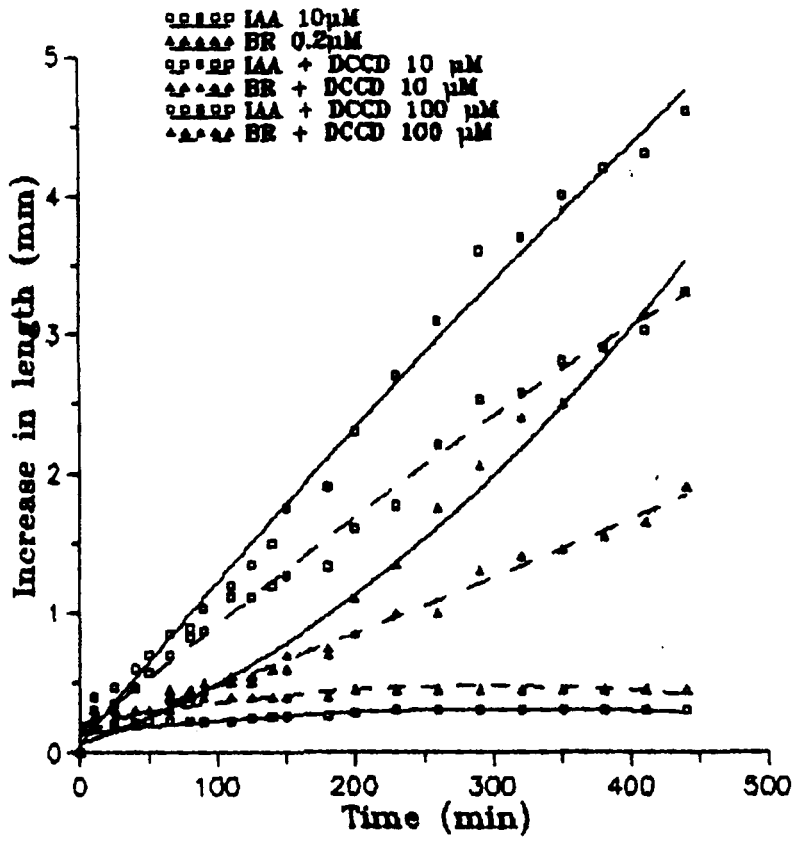


Fig. 23b: Time course study on concentration effect of DCCD on IAA and BR induced growth in wheat coleoptile.

[Coleoptile segments were incubated in IAA (1Ø μM) or BR (Ø.2 μM) medium with different concentrations of DCCD. The coleoptile length were measured at different intervals after floating over the medium.]

minutes after incubation period. The inhibitory effect of DCCD on BR induced growth was observed after 120 minutes. However, at high concentration of DCCD (100 μM), both IAA and BR induced growth was inhibited from the time of addition of DCCD. These results indicate that there is a significant difference in the effect of DCCD on IAA and BR induced growth. DCCD at a lower concentration was effective in inhibiting the IAA induced growth as early as 90 minutes after incubation with DCCD. BR induced elongation responses were observed only after 120 minutes of incubation and DCCD effect was also observed starting from 120 minutes.

These results also indicate that, both the metabolic inhibitors (KCN and DCCD) were effective in inhibiting IAA and BR induced elongation growth.

4.3.2.3 EFFECT OF RNA AND PROTEIN SYNTHESIS INHIBITORS ON IAA AND BR INDUCED ELONGATION GROWTH OF COLEOPTILE SEGMENTS

Auxin induced cell elongation of excised plant tissues have been shown that continued mRNA and protein synthesis is essential for elongation responses to be sustained for a long period. To know whether BR stimulated cell elongation also needs mRNA and protein synthesis, the influence of RNA and protein synthesis inhibitors on BR induced elongation was studied.

4.3.2.3.1 Influence of continuous presence of inhibitors on IAA and BR induced growth

Coleoptile segments were incubated in IAA (10 μM) or BR (0.2 μM) with different concentrations of inhibitors. Final length of coleoptile segments were measured after 24 hours of incubation.

5-Fluorouracil (5-FLU): 5-FLU was used as an inhibitor of RNA biosynthesis. Both IAA and BR induced growth was inhibited by 5-FLU. At 10 μM concentration of 5-FLU, there was more than 60 per cent inhibition of both IAA and BR induced growth (Fig. 24). For instance, at 10 μM of concentration of 5-FLU, growth was inhibited to an extent of 66 per cent in both IAA and BR medium. With further increase in concentration of 5-FLU the inhibitory effect on growth was also increased. At 1 mM concentration of 5-FLU, both IAA and BR induced growth was inhibited completely .

Cycloheximide (CH): Cycloheximide an inhibitor of cytoplasmic protein synthesis, inhibited both IAA and BR induced elongation growth as shown in Fig. 25. Increasing concentration of cycloheximide in the incubation medium, progressively reduced IAA and BR stimulated elongation of coleoptile segments. At a concentration of 10 μM cycloheximide the IAA and BR induced growth was inhibited by 55 and 60 per cent, respectively.

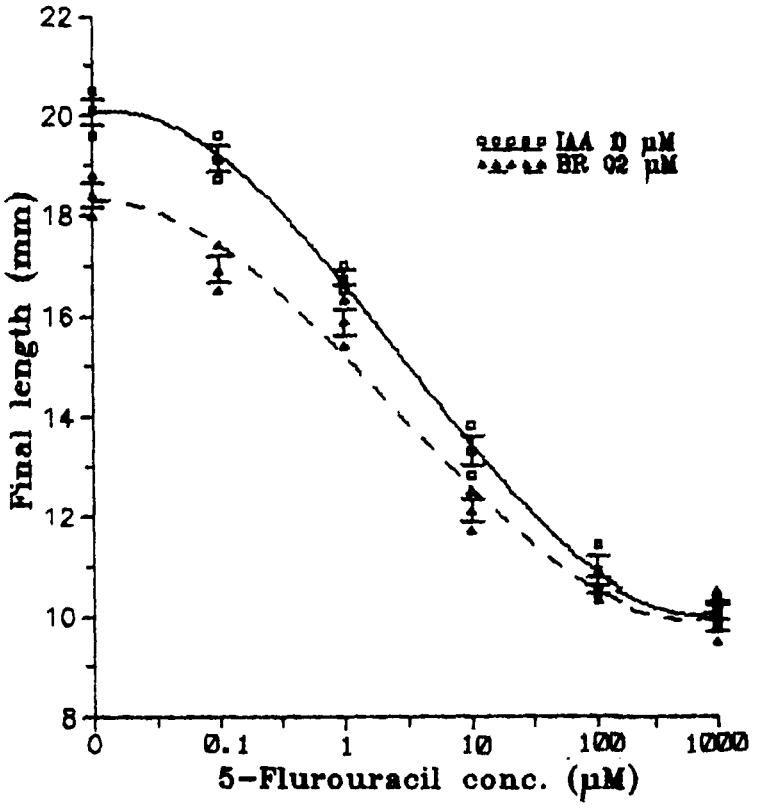


Fig. 24: Effect of RNA synthesis inhibiotor 5-FLUOROURACIL on IAA and BR induced growth of wheat coleoptile segments.

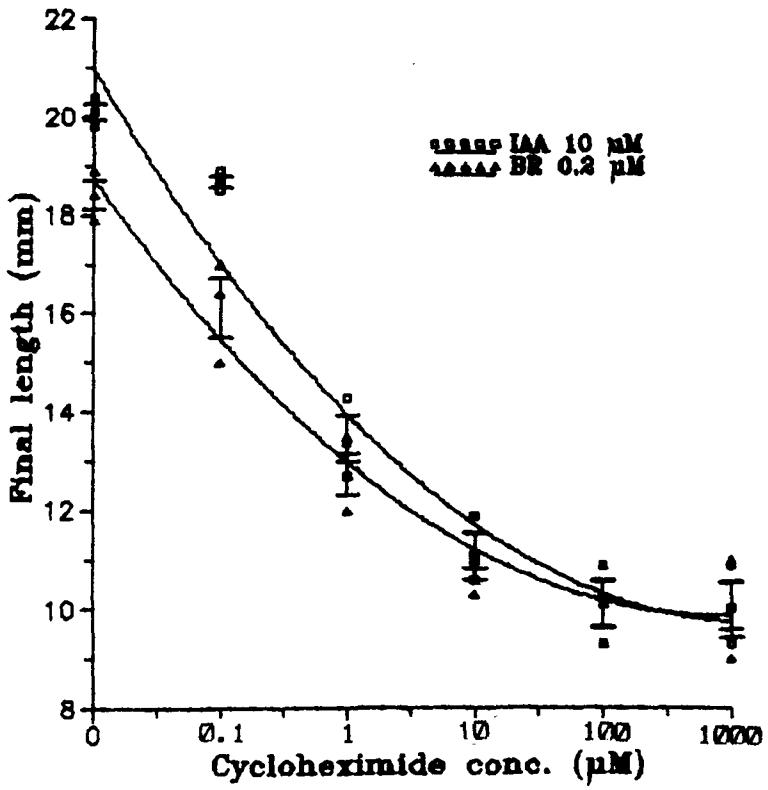


Fig. 25: Effect of protein synthesis inhibitor CYCLOHEXIMIDE on IAA and BR induced growth of wheat coleoptile segments.

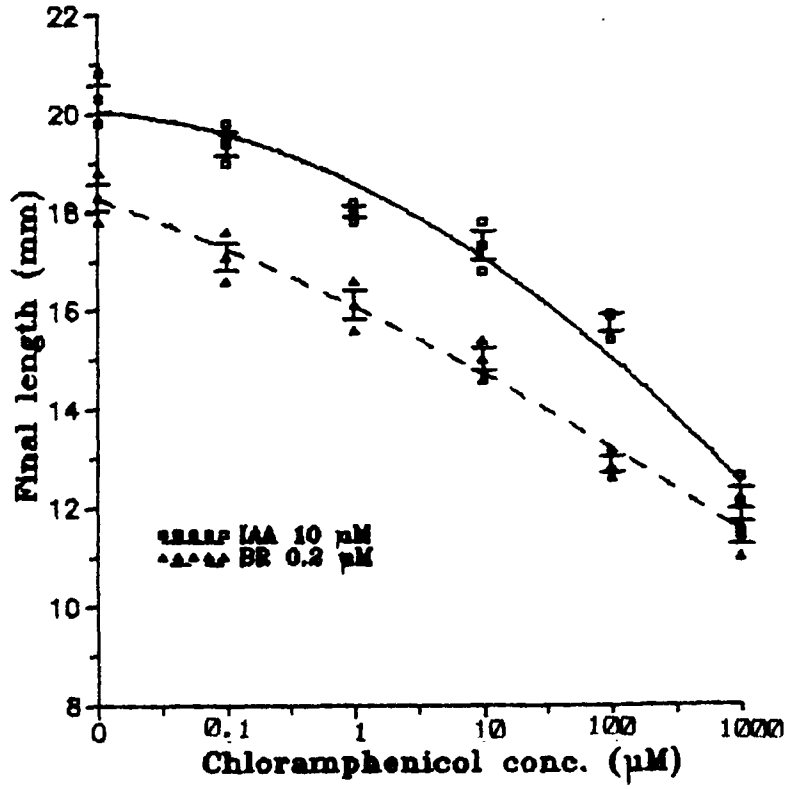


Fig. 26: Effect of protein synthesis inhibitor CHLORAMPHENICOL on IAA and BR induced growth of wheat coleoptile segments.

Chloramphenicol: In the presence of chloramphenicol along with IAA and BR, a concentration dependent decrease in coleoptile growth was observed. As shown in Fig. 26, the inhibitory effect of chloramphenicol on IAA and BR induced growth was more pronounced when the concentration was more than 100 μM.

4.3.2.3.2 Influence of pretreatment of coleoptile segments with RNA and Protein synthesis inhibitors on IAA and BR induced growth

In another set of experiments, the influence of pretreatment effect of RNA and protein synthesis inhibitors on IAA and BR induced growth was studied. Tables 4a and 4b show the duration of treatment and the final length of coleoptile segments. Coleoptile segments were pretreated with 5-FLU (10 μM) or CH (10 μM), for a duration of 2 or 4 hours and then transferred to IAA or BR medium with and without 5-FLU (10 μM) and CH (10 μM). Final length of the coleoptiles were measured 24 hours after transferring to IAA or BR medium.

5-Fluorouracil: Pretreatment of coleoptile segments with 5-FLU (10 μM) completely inhibited IAA and BR induced growth. Pretreatment for 2 hours inhibited the growth to an extent of 53 and 57 per cent in IAA, and BR respectively along with 5-FLU in the media.

Cycloheximide: Cycloheximide pretreatment also inhibits both IAA and BR induced elongation growth of

Table 4a : Effect of pretreatment with 5-FLUOROURACIL and CYCLOHEXIMIDE on IAA stimulated growth of wheat coleoptile segments.

Pretreatment	Post treatment (24 hours)	Final length of coleoptile (mm)
---	IAA(10 µM)	20.4 ± 0.2
5-Fluorouracil(5-FLU)		
2 hours	IAA(10 µM)+5FLU(10 µM)	10.8 ± 0.1
4 hours	IAA(10 µM)+5FLU(10 µM)	10.1 ± 0.1
Cycloheximide(CH)		
2 hours	IAA(10 µM)+CH(10 µM)	10.4 ± 0.1
4 hours	IAA(10 µM)+CH(10 µM)	10.0 ± 0.1
CD at 1 %		2.1 ± 0.05

Table 4b : Effect of pretreatment with 5-FLUOROURACIL and CYCLOHEXIMIDE on BR stimulated growth of wheat coleoptile segments.

Pretreatment	Post treatment (24 hours)	Final length of coleoptile (mm)
---	BR(0.2 µM)	18.3 ± 0.2
5-Fluorouracil(5-FLU)		
2 hours	BR(0.2 µM)+5FLU(10 µM)	10.4 ± 0.1
4 hours	BR(0.2 µM)+5FLU(10 µM)	10.0 ± 0.1
cycloheximide(CH)		
2 hours	BR(0.2 µM)+CH(10 µM)	10.4 ± 0.1
4 hours	BR(0.2 µM)+CH(10 µM)	10.0 ± 0.1
CD at 1 %		2.0 ± 0.05

coleoptile segments. Coleoptile segments pretreated for 2 hours in cycloheximide inhibited the growth to an extent of 51 and 57 per cent in IAA + CH (10 μM) and BR + CH (10 μM) media, respectively. These results indicate that IAA and BR induced elongation of coleoptile tissue involves increase in RNA and protein synthesis.

4.3.3 INFLUENCE OF ADENOSINE PHOSPHATE ON IAA AND BR INDUCED GROWTH IN COLEOPTILE SEGMENTS

Experiments were conducted to study the influence of metabolic energy source on IAA and BR induced elongation of coleoptile segments. Table 5a shows the addition of AMP, ADP and ATP. IAA induced elongation was significantly more in the presence of adenosine triphosphate (ATP). At higher concentration of ATP used (500 μM), 15 per cent increase in elongation was observed over IAA alone. Similarly, 8 per cent increase in BR induced elongation at 500 μM concentration of ATP was noticed.

In another experiment, the influence of pretreatment effect of ATP on IAA and BR induced elongation was assessed (Table 5b). Pretreating the coleoptile segments for 30 and 60 minutes with 500 μM ATP resulted in marked increase in elongation of coleoptile segments. The results of above experiments indicated that both IAA and BR resulted in marked increase in growth in the presence of ATP as an energy source.

Table 5a : Interaction of IAA and BR with adenosine phosphates in elongation growth of wheat coleoptile segments.

(coleoptile length in mm)

Treatment (conc. µM)	Water	AMP (µM)		ADP (µM)		ATP (µM)	
		100	500	100	500	100	500
Water	15.8	16.1	16.1	16.0	16.2	16.3	16.3
IAA 10 µM	18.9	18.8	19.1	19.0	19.0	20.1	20.3
BR 2 µM	17.8	18.1	18.3	18.1	18.2	19.3	19.3

Table 5b : Pretreatment of coleoptile segments with ATP on IAA and BR induced elongation growth of wheat coleoptile segments.

(coleoptile length in mm)

Treatment	Water	ATP concentrations (µM)			
		100		500	
		30 min	60 min	30 min	60 min
WATER	16.9	16.3	16.1	16.7	16.7
IAA 10 µM	19.7	20.3	20.3	21.0	20.6
BR 2 µM	18.4	18.4	19.1	18.8	19.4

4.3.4 THE ROLE OF BR IN AUXIN BIOSYNTHESIS IN COLEOPTILE SEGMENTS

BR induced growth responses are similar to IAA in many test systems. BR might be influencing endogenous auxin concentration and thus influencing elongation. This hypothesis was tested using coleoptile tips, which contains all the enzymes required for auxin biosynthesis. In this set of experiments, coleoptile tips were incubated in the presence of precursor of auxin-tryptophan with and without GA and BR.

Incubation of coleoptile tips in presence of auxin resulted in an increase in growth of tip segments. However, the increase was less. In the presence of GA (10 μM) and Tryptophan, there was a marked increase in the growth of coleoptiles (Fig. 27a). Presence of BR along with Tryptophan also resulted in marked increase in the final length of tip segments (Fig. 27b).

Addition of inhibitor, Dimedone (indole acetaldehyde to IAA) to the medium, completely inhibited both GA and BR induced elongation of coleoptile tip segments in the presence of Tryptophan (Fig. 27c). However, Dimedone was ineffective in inhibiting IAA induced growth (Fig. 27d).

These experiments suggest that both GA and BR increase elongation of coleoptile tips by increasing conversion of tryptophan to IAA.

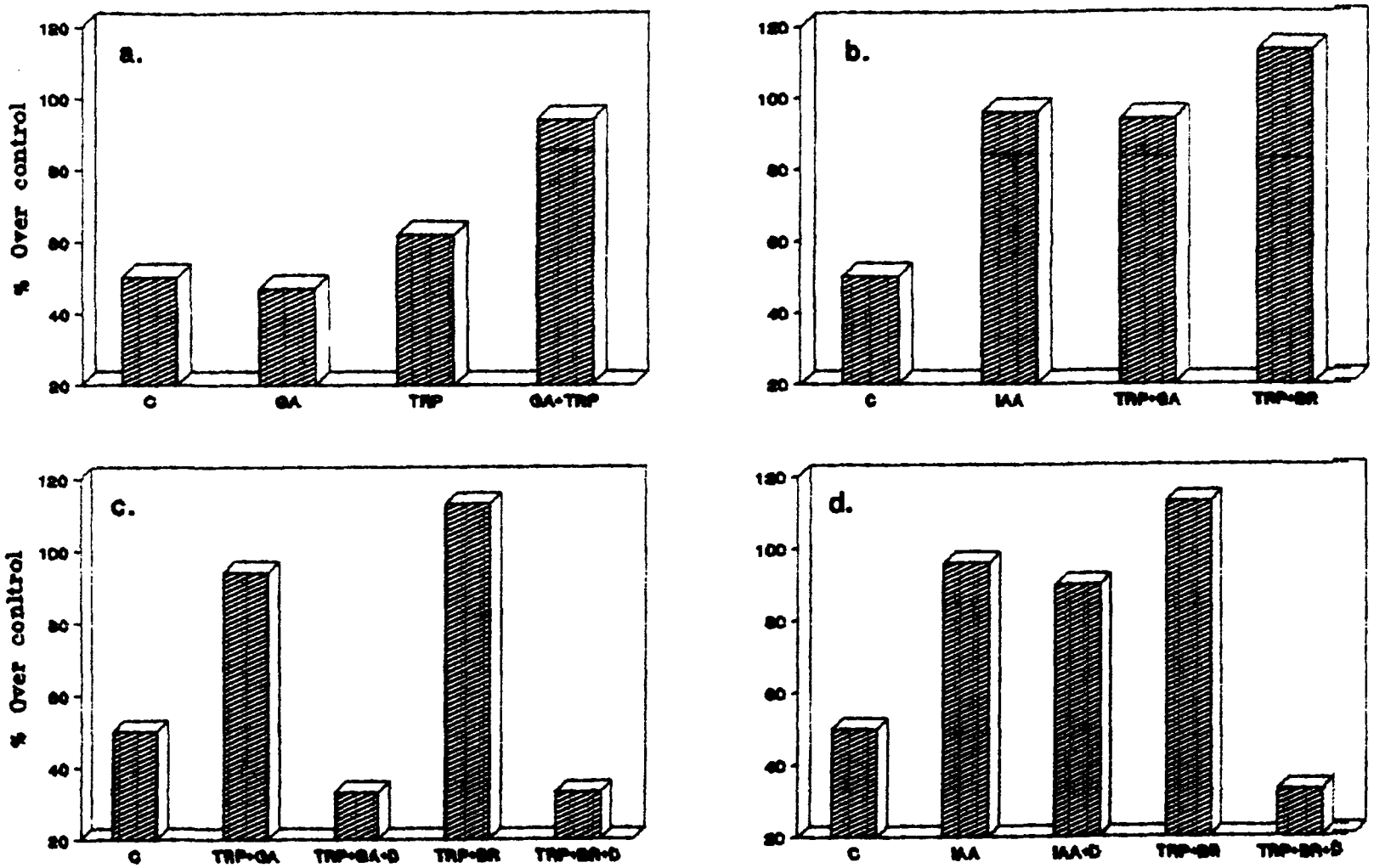


Fig. 27: Growth of coleoptile tip segments in the presence of Trp, BR, and GA with or without auxin synthesis inhibitor. a) influence of GA with Trp, b) comparative effect of GA and BR with Trp, and c&d) influence of auxin biosynthesis inhibitor on GA and BR induced growth with Trp.

[C=Control, Trp=Tryptophan (10 μM), GA=Gibberellic acid (10 μM), BR=Brassinolide (0.2 μM), IAA=Indole-3-acetic acid (10 μM), and D=Dimedon (10 μM)]

4.3.5 ESTIMATION OF IAA IN COLEOPTILE SEGMENTS BY IMMUNOASSAY TECHNIQUE

One of the important objectives of the research work was to see the mode of action of BR in induction of growth responses. Many workers have suggested that BR induces growth responses by increasing biosynthesis of IAA. In this set of experiments, the influence of BR on IAA biosynthesis was studied. To accomplish this polyclonal antibodies were raised against IAA.

4.3.5.1 IAA-BSA CONJUGATION AND SPECIFIC ANTIBODY ASSAY

IAA was conjugated to BSA, the carrier protein using the carboxyl site. Determination of binding ratio showed that an average of 7 to 8 molecules of IAA were bound to each molecule of BSA (Table 6).

Immunodiffusion technique showed the precipitation pattern of IAA-BSA antisera (Plate 5). The thickness of the precipitation bands corresponding to the wells containing the IAA-BSA conjugate was more compared to BSA alone. The bands developed against the conjugate was concentration dependent. This suggested that the immunized rabbits had responded positively to hapten conjugate and produced specific antibodies for hapten and also for carrier protein BSA.

Further, specificity and titer was confirmed by quantitative precipitation test and membrane binding

Table 6 : Determination of coupling ratios for IAA-BSA conjugate.

Optical density	IAA-BSA conjugate
Absorbance of conjugate at 266 nm	1.71
Absorbance of BSA at 266 nm	0.49
Difference in absorbance	1.22
Conjugation ratio	8

assay methods. The titer of precipitable antibody was 1.8 $mg.ml^{-1}$ (Fig. 28). The test also revealed that the specific antibody against the carrier protein was also present at a relatively low level of 0.5 $mg.ml^{-1}$. This showed that at least 200 per cent of the IgG was specific to the hapten over control.

The membrane binding assay, also confirmed the presence of specific antibodies against the hapten in the IAA-BSA antisera (Table 7). An average of 708 cpm was retained by the filter in buffer alone or in the presence of normal rabbit serum. In the presence of the anti IAA-BSA antisera about 1668 cpm was bound over control.

4.3.5.2 STANDARD CURVE FOR IAA USING INDIRECT ELISA

The presence of free IAA in plant extracts from different experiments was determined in an indirect competitive binding ELISA. Standard curve was developed with 0.02-1000 pmoles.0.1 ml^{-1} of the standard IAA. 0.1 ml of a 1:20000 dilution of the primary antibody (IAA-BSA IgG) was mixed with different concentrations of 0.1 ml of standard IAA before being added to the coated microtiter wells. Free IAA will bind to the IAA specific antibodies in the solution and prevent their subsequent binding by competition inhibition, to the IAA attached to the plate, though the carrier protein casein BSA specific antibodies in the antiserum are eliminated during this process, as

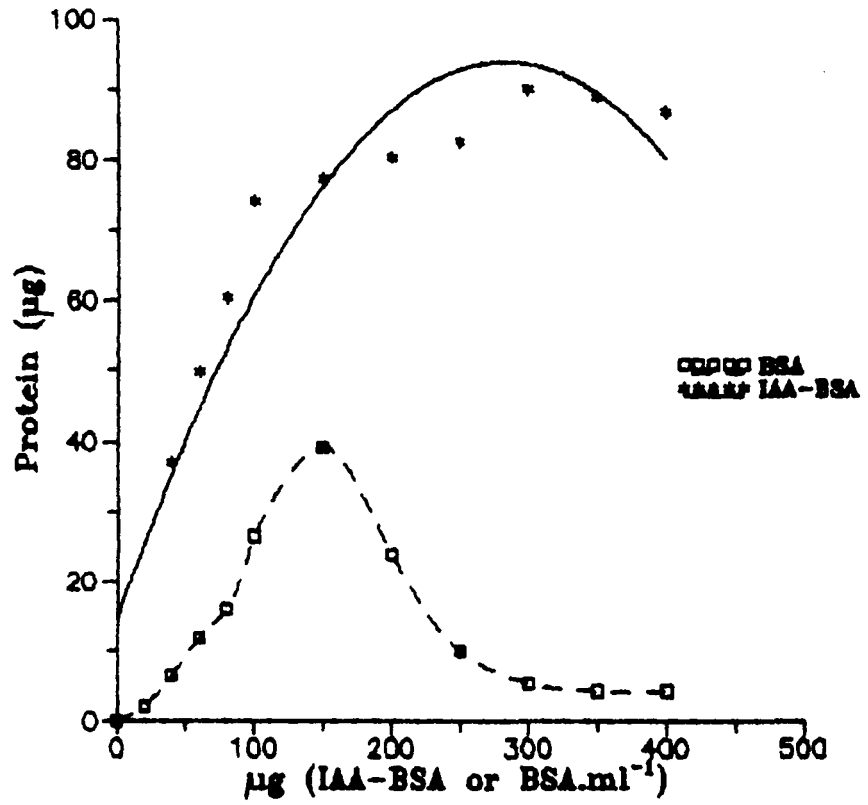


Fig. 28 : Quantitative precipitation analysis of IAA-BSA polyclonal antibodies.

Table 7: Membrane binding assay for detection of specific antibodies against hapten (free IAA) in antiserum raised against IAA-BSA conjugate.

Treatment	Radioactivity on membrane (cpm)
*Control	708
IAA-BSA serum	2376

* Serum from nonimmunised animal

the IAA (antigen) is coated to the polystrene plate using a different protein. In this standard curve B/B_o representing binding ratio for each concentration of standard IAA was determined and was converted to a linear logit B/B_o function using the equation,

Logit B/B_o = Ln (B/B_o %) / (100-B/B_o %)

The standard curve using this indirect ELISA technique is presented in Fig. 29. Here the intensity of the colour is inversely proportional to the concentration of IAA added. Therefore a negative linear relationship was obtained with the highest concentration of IAA (800 pmoles.assay^{-1}) giving a logit B/B_o of -2.03 and the lowest concentration (0.02 pmoles.assay^{-1}) giving a value of +3.03.

4.3.5.3 INFLUENCE OF BR AND GA ON IAA BIOSYNTHESIS IN COLEOPTILE TIP SEGMENTS

Coleoptile tip segments (10 mm) were incubated in tryptophan and other cofactors with and without BR, GA and incubated in dark for 4,8 and 12 hours. After different durations of incubation, coleoptiles were ground and IAA content was determined by indirect ELISA. In the presence of BR, BR+Trp, and GA+Trp there was marked increase in IAA content at 4, 8, and 12 hours and IAA levels were maintained till 8 hours (Table 8). At 12 hours after incubation IAA content was relatively less in BR and BR+Trp treatment. But in GA+Trp the IAA content was maintained high even at 12 hours treatment.

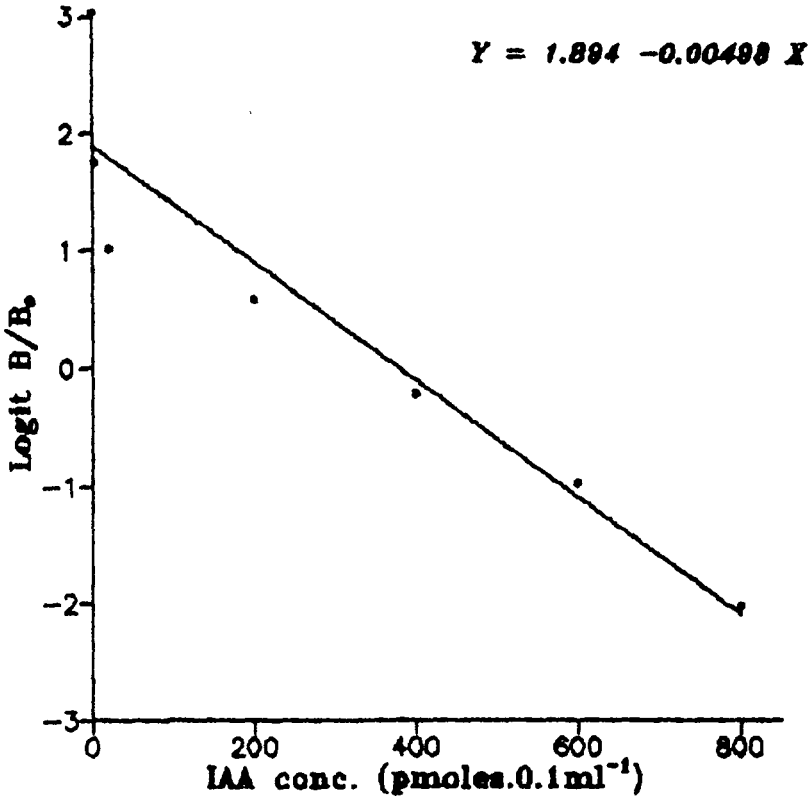


Fig. 29: Standard curve of IAA using indirect ELISA.

Table 8: Influence of BR and GA on IAA biosynthesis in coleoptile tip segments.

(nmoles.20 coleoptiles^{-1})

Treatment	4 hrs	8 hrs	12 hrs
Control	2.65	4.66	3.06
Tryptophan	3.30	4.88	3.90
BR	12.66	12.13	7.71
BR + Tryptophan	14.17	11.30	8.13
GA + Tryptophan	11.42	10.29	10.03

This suggests that both BR and GA increases IAA content in coleoptile tips by increasing conversion of tryptophan to IAA.

4.3.6 INFLUENCE OF BR ON BINDING OF ^{14}C IAA TO MEMBRANES AND AUXIN BINDING PROTEINS (ABPs)

Experiments conducted with coleoptile tips indicated that, the growth of coleoptile was always more in medium containing both IAA and BR compared to the medium containing either BR or IAA. Pretreatment of coleoptile tissue with BR followed by transferring the tissue to IAA also resulted in more cell elongation compared to IAA to BR. These results indicated that BR might be increasing the receptivity of tissue for IAA or increasing the binding of IAA to the specific receptor proteins. This was analysed using radioactive labelled IAA (^{14}C IAA).

In first experiment, coleoptile tissues were incubated in medium containing ^{14}C IAA + cold IAA and ^{14}C IAA + cold IAA + BR. At the end of 24 hrs of incubation period, the coleoptile tissue was analysed for ^{14}C activity in membrane fractions.

The activity in different fractions of coleoptiles were measured in 12 and 24 hrs after incubating in test solutions. The cytosol fraction contains more activity followed by cell wall fractions and membrane fractions (Table 9). The ^{14}C activity was more in IAA + BR

Table 9: ^{14}C activity in various fractions from the coleoptile incubated in ^{14}C IAA with IAA (10 μM), and IAA + BR at the end of 12 hrs and 24 hrs incubation period.

(CPM.20coleoptile^{-1})

Treatments	12 hrs incubation				24 hrs incubation			
	cell wall	membrane	cytosol	total	cell wall	membrane	cytosol	total
IAA	384	98	2310	2792	1024	121	3693	4838
IAA+BR	383	129	2511	3023	1337	145	4121	5603

compared to IAA in all fractions. The per cent increase in total radioactivity in the presence of IAA + BR compare to IAA alone was 199 per cent at 12 hrs after incubation and 116 per cent at 24 hrs after incubation. The per cent increase in activity in membrane fraction in IAA + BR treatment over IAA treatment alone was 132 and 120 per cent at 12 and 24 hrs of incubation respectively. The results indicated that binding of IAA to the membrane fraction was increased in presence of IAA and BR together, suggesting that, BR is increasing the binding of IAA to membrane fractions of the tissue.

To study the effect of BR on binding of IAA to IAA receptor proteins, auxin binding proteins (ABPs) were isolated and were incubated in medium containing ^{14}C IAA with different concentrations of BR. Presence of BR in the medium increased binding of ^{14}C IAA to both IAA specific and non specific proteins (Fig. 30a and 30b). With an increase in concentration of BR , specific binding increased up to the concentration of 10 µM BR. However, non specific binding increased only up to 2 µM of BR and, further increase in BR,reduced the non-specific binding. A marked increase in specific binding was observed with increase in concentration of BR. These results indicated that BR increases binding of auxins to ABPs.

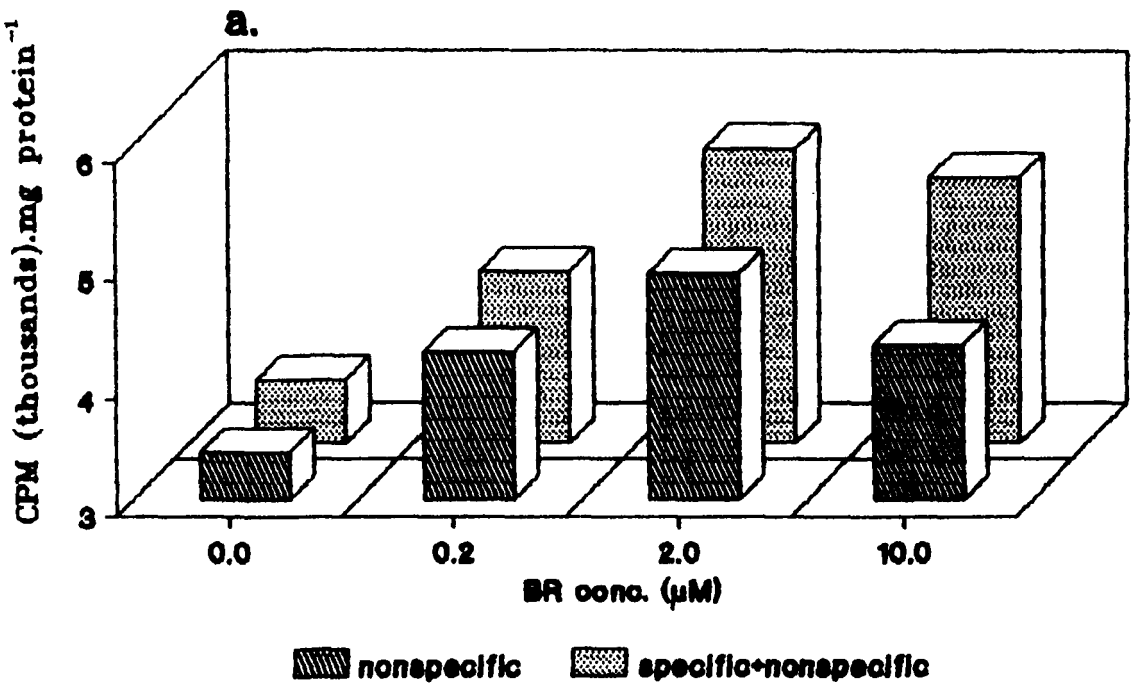


Fig. 30: Influence of different concentrations of BR on ^{14}C IAA binding to ABPs isolated from wheat coleoptile tissue.
a) Nonspecific and specific + nonspecific, and
b) Specific binding

4.4 ROLE OF CALCIUM IN IAA AND BR INDUCED ELONGATION GROWTH OF COLEOPTILE SEGMENTS

The hormonal regulation of plant growth and development was shown to be influenced by the presence of calcium. Auxin induced growth responses in coleoptile segments were also shown to be mediated by calcium release to cytosol and calcium modulated protein-calmodulin.

In this series of experiments, the role of calcium and calmodulin in IAA and BR induced elongation growth of coleoptile segments were studied.

The role of calcium in growth responses was studied by altering the concentration of calcium in the cytosol. The resulting change in growth responses brought about by modified calcium concentration in the presence of hormones can be attributed to changes in cytosolic calcium concentrations.

Calcium concentration in the cytosol can be reduced by using specific chemicals which would block calcium entry into cytosol or the chemicals which inhibit calcium action directly in the cytosol. The other method to modify cytosolic calcium concentration in the tissue is by raising the seedlings in calcium enriched medium.

In this investigation, cytosolic calcium concentration was modified by using specific chemicals

which reduce free calcium concentrations in the tissue as well as by growing the seedlings in calcium enriched medium, so that the calcium concentration in the tissue will be higher.

4.4.1 EXPERIMENTS WITH WATER GROWN TISSUE

4.4.1.1 EXPERIMENTS USING EGTA

Ethylene glycol-bis(aminoethyl) tetraacetic acid (EGTA) is a specific chelator of calcium.

Wheat coleoptile segments were incubated in basal growth medium containing optimum concentration of IAA or BR with different concentrations of EGTA, for 24 hours. At the end of incubation period, final length of coleoptile segments were measured.

Presence of EGTA along with IAA or BR significantly reduced the growth. With increasing concentration of EGTA in the medium, a progressive reduction in coleoptile length was observed. At 5 mM of EGTA concentration, maximum inhibition was observed (Fig. 31a).

In another set of experiments, the effect of pretreatment of coleoptile segments with EGTA (10 mM) on their growth in water, IAA, and BR medium was examined. Coleoptile segments were pretreated in EGTA for 4 hours and then transferred to growth medium in presence or absence of IAA or BR for another 24 hours. The final

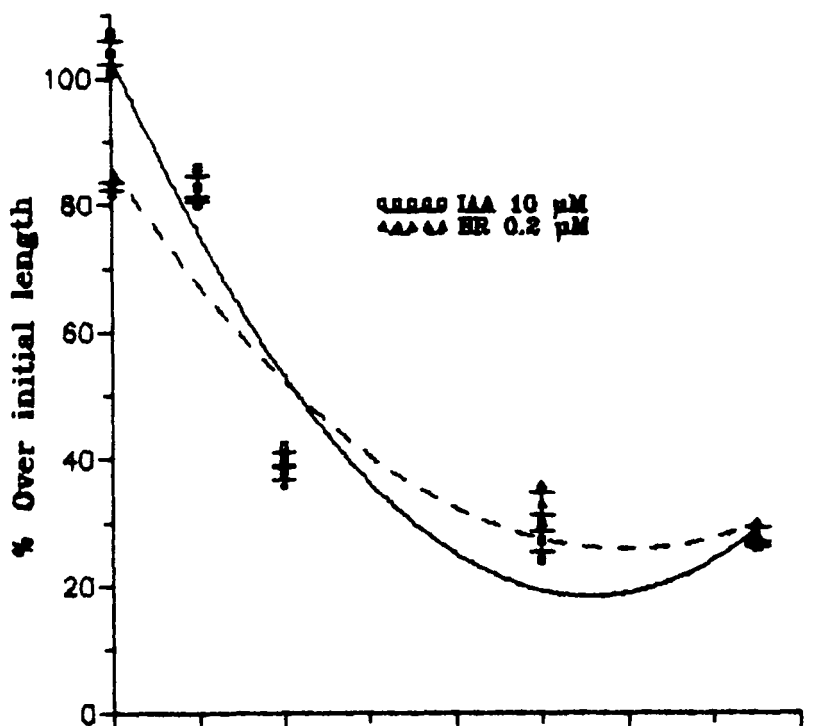


Fig. 31a: Effect of different concentrations of EGTA on IAA and BR induced growth in wheat coleoptile segments.

[Coleoptile segments were incubated in medium containing IAA (1Ø µ M) or BR (Ø.2 µ M) with different concentrations of EGTA. Final length of coleoptile segments were measured at the end of 24 hours incubation period]

length of the coleoptile segments was measured at the end of incubation period (Fig. 31b).

In the presence of IAA and BR, growth of coleoptile segments were increased by 116 and 108 per cent respectively compared to 65 per cent in basal medium. EGTA pretreatment resulted in only 48, 58 and 53 per cent increase in growth in basal, IAA and BR medium respectively. This indicates that the IAA and BR induced growth reduces by more than 50 per cent when the coleoptile segments were pretreated with EGTA.

4.4.1.2 EXPERIMENTS USING LANTHANUM CHLORIDE

The role of lanthanum chloride in IAA and BR induced elongation growth of coleoptile segments was examined by adopting the protocol described earlier.

When Lanthanum chloride was present in the medium a concentration dependent reduction in coleoptile segment elongation was observed. Lanthanum chloride at 10 nM concentration level reduced the IAA and BR induced elongation to an extent of 78 and 74 per cent, respectively over IAA and BR alone (Fig. 32a).

Pretreatment of coleoptile segments for 4 hours with lanthanum chloride (1 and 10 nM) prior to transfer to IAA and BR medium also resulted in significant reduction in length of coleoptile segments (Fig. 32b).

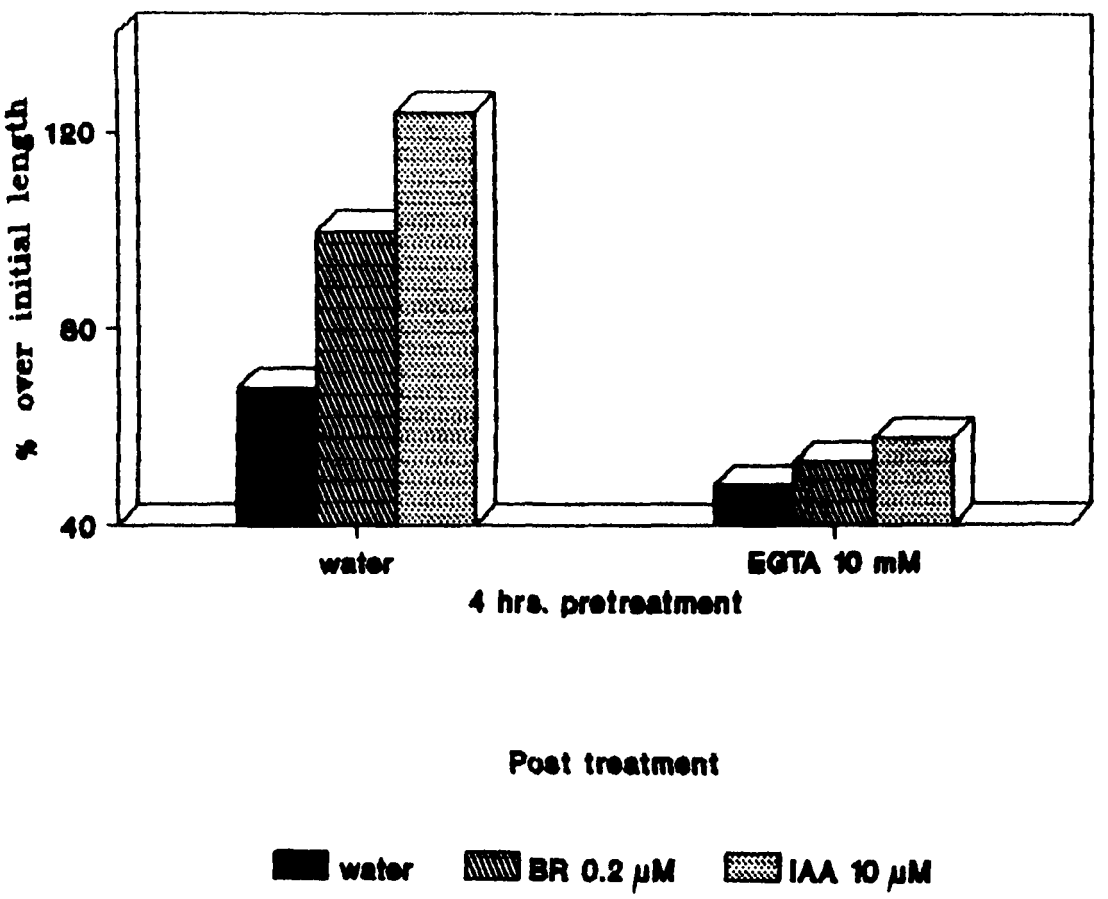


Fig. 31b: Influence of pretreatment of coleoptile segments with EGTA on IAA and BR induced growth.

[Coleoptile segments were pretreated with water or EGTA (1Ø mM) for a period of 4 hours and then transferred to water or IAA (1Ø μ M) or BR (Ø.2 μ M) medium. Final length of segments were measured after 24 hours of post treatment.]

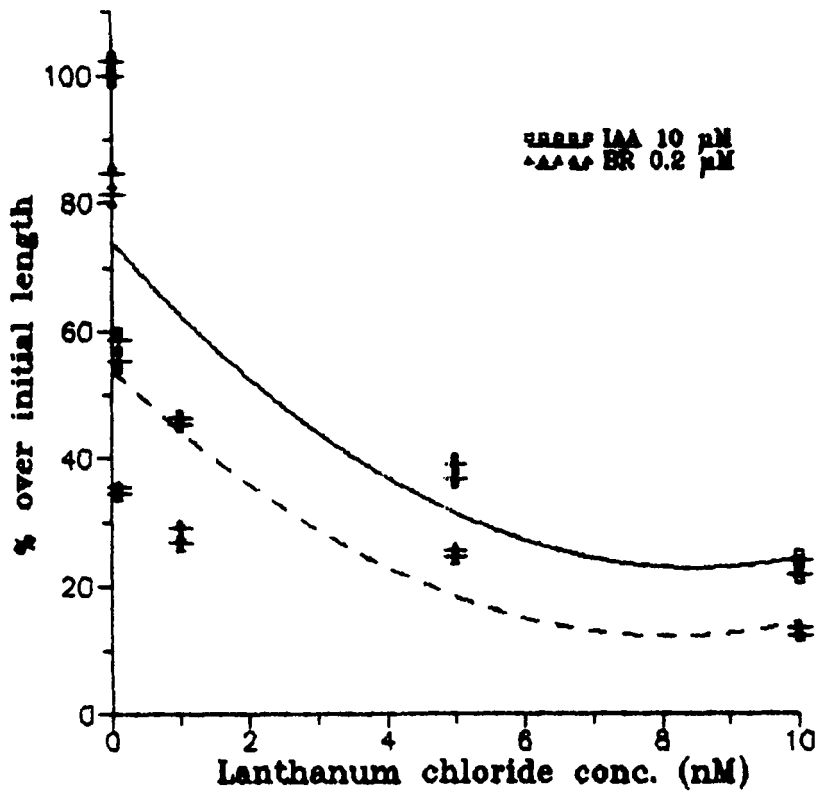


Fig. 32a: Effect of different concentrations of of LANTHANUM CHLORIDE on IAA and BR induced growth in wheat coleoptile segments.

[Coleoptile segments were incubated in medium containing IAA (1Ø μ M) or BR (Ø.2 μ M) with different concentrations of lanthanum chloride. Final length of coleoptile segments were measured at the end of 24 hours incubation period.]

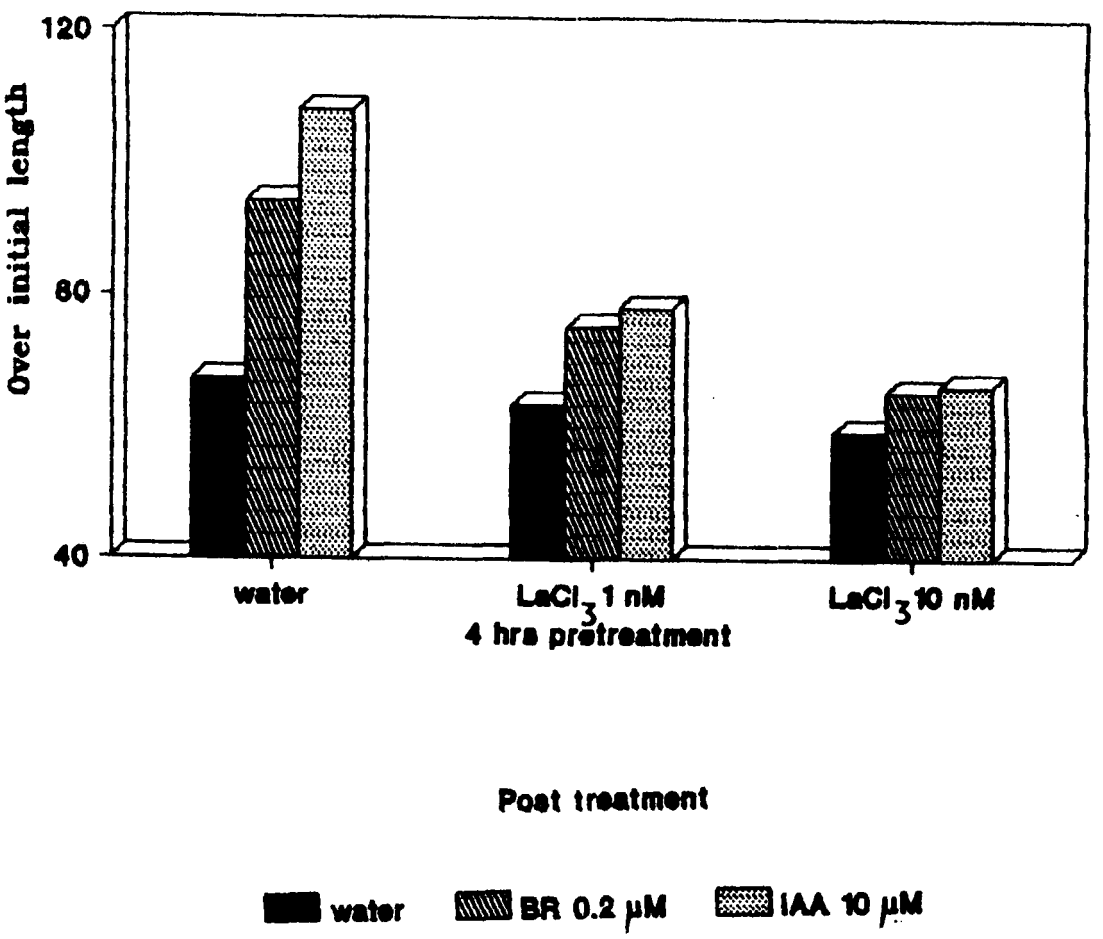


Fig. 32b: Influence of pretreatment of coleoptile segments with lanthanum chloride on IAA and BR induced growth.

[Coleoptile segments were pretreated with water or different concentrations of $LaCl_3$ (1 and 10 μ M) for a period of 4 hours and then transferred to water or IAA (10 μ M) or BR (0.2 μ M) medium. Final length of segments were measured after 24 hours of post treatment.]

4.4.1.3 EXPERIMENT WITH CHLORPROMAZINE (CPZ)

The role of CPZ on IAA and BR induced elongation of coleoptile segments was investigated. Coleoptile segments were incubated in basal, IAA (10 μM) or BR (0.2 μM) medium with different concentrations of CPZ. The final length of coleoptile segments were recorded after 24 hours incubation in the medium.

Presence of CPZ at a concentration of 50 μM or more resulted in significant reduction in elongation (Fig. 33a). Increasing concentration of CPZ in the incubation medium showed a linear reduction in the growth of coleoptile in the basal, IAA, and BR media. At 150 μM concentration of CPZ, the final length of coleoptile segments remained same in basal, IAA and BR media.

The role of pretreatment of coleoptile segments with CPZ on IAA and BR induced elongation growth was also examined (Fig.33b). When coleoptile segments were pretreated at different concentrations of CPZ, IAA and BR induced growth reduced significantly. There was concentration dependent reduction in coleoptile growth due to pretreatment with CPZ. For example, segments incubated in IAA (10 μM) medium showed 133 per cent increase in growth. However, when the segments were pretreated with 50, 100 or 150 μM of CPZ the increase were 105, 80 and 64 per cent respectively. Similar trend was observed when

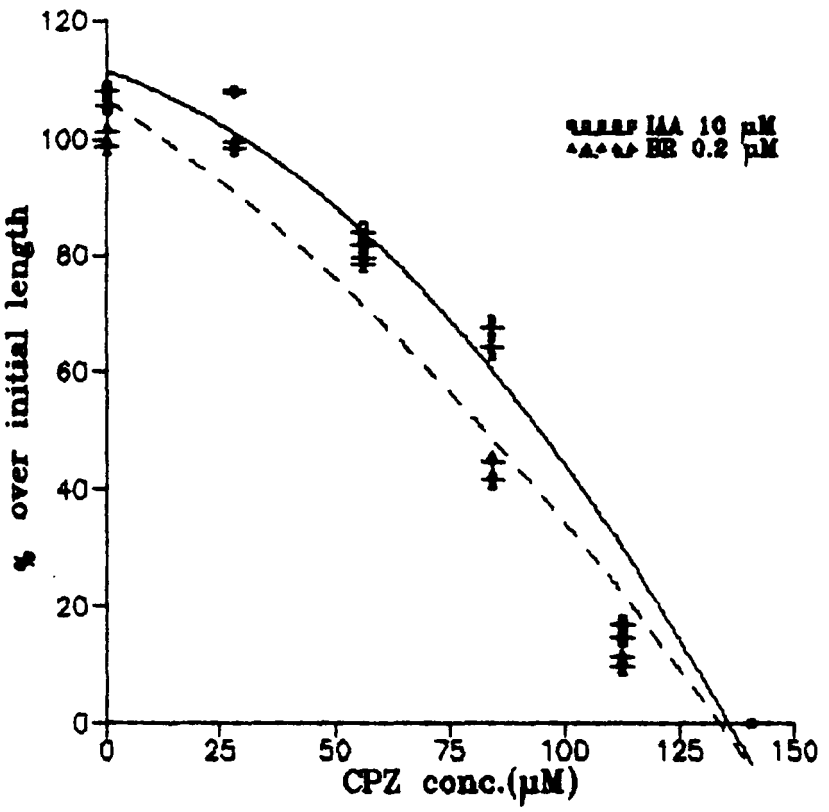


Fig. 33a: Effect of different concentrations of CPZ on IAA and BR induced growth in wheat coleoptile segments.

[Coleoptile segments were incubated in medium containing IAA (1Ø µ M) or BR (Ø.2 µ M) with different concentrations of CPZ. Final length of coleoptile segments were measured at the end of 24 hours incubation period.]

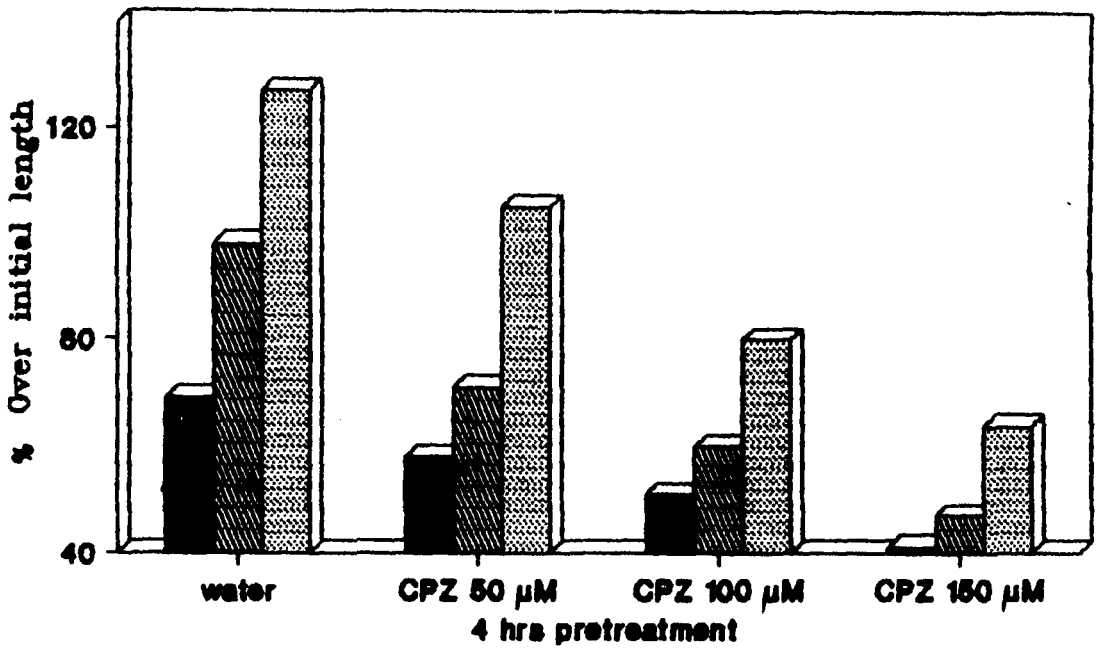


Fig. 33b: Influence of pretreatment of coleoptile segments with CPZ on IAA and BR induced growth.

[Coleoptile segments were pretreated with water or different concentrations of CPZ (5Ø, 1ØØ and 1Ø µM) for a period of 4 hours and then transferred to water or IAA (1Ø µM) or BR (Ø.2 µM) medium. Final length of segments were measured after 24 hours of post treatment.]

the CPZ pretreated coleoptile segments were transferred to BR (0.2 μM) medium.

4.4.1.4 EXPERIMENTS USING RUTHENIUM RED

The role of Ruthenium Red (RR) on IAA and BR induced elongation growth of coleoptile segments was studied by incubating in medium containing IAA and BR with and without RR. Ruthenium Red at a concentration of 5 and 10 μM caused significant reduction in IAA and BR induced growth. At 10 μM concentration of RR the inhibitory effect on growth was more compared to 5 μM concentration of RR (Fig. 34a).

In another set of experiments, the influence of pretreatment of coleoptile segments with RR (4 hrs) on IAA and BR induced growth was examined (Fig. 34b). Coleoptile segments pretreated with 20 and 30 μM of RR showed significant reduction in growth when transferred to basal, IAA and BR media. For example, coleoptile segments in IAA (10 μM) and BR (0.2 μM) showed the growth of 107 and 90 per cent,respectively. However, segments pretreated with 30 μM of RR, showed only 65 and 59 percent growth in IAA and BR media respectively.

4.4.1.5 EXPERIMENTS USING VERAPAMIL

The role of verapamil on IAA and BR induced growth of coleoptile segments was examined. Coleoptile segments

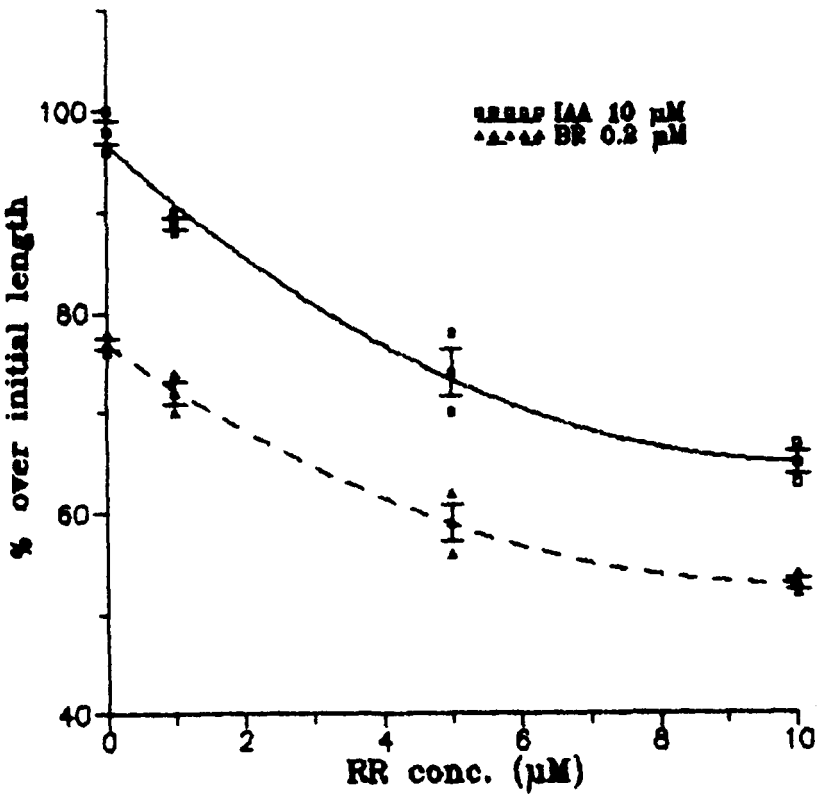


Fig. 34a: Effect of different concentrations of RR on IAA and BR inducde growth in wheat coleoptile segments.

(Coleoptile segments were incubated in medium containing IAA (1Ø µ M) or BR (Ø.2 µ M) with different concentrations of RR. Final length of coleoptile segments were measured at the end of 24 hours incubation period.)

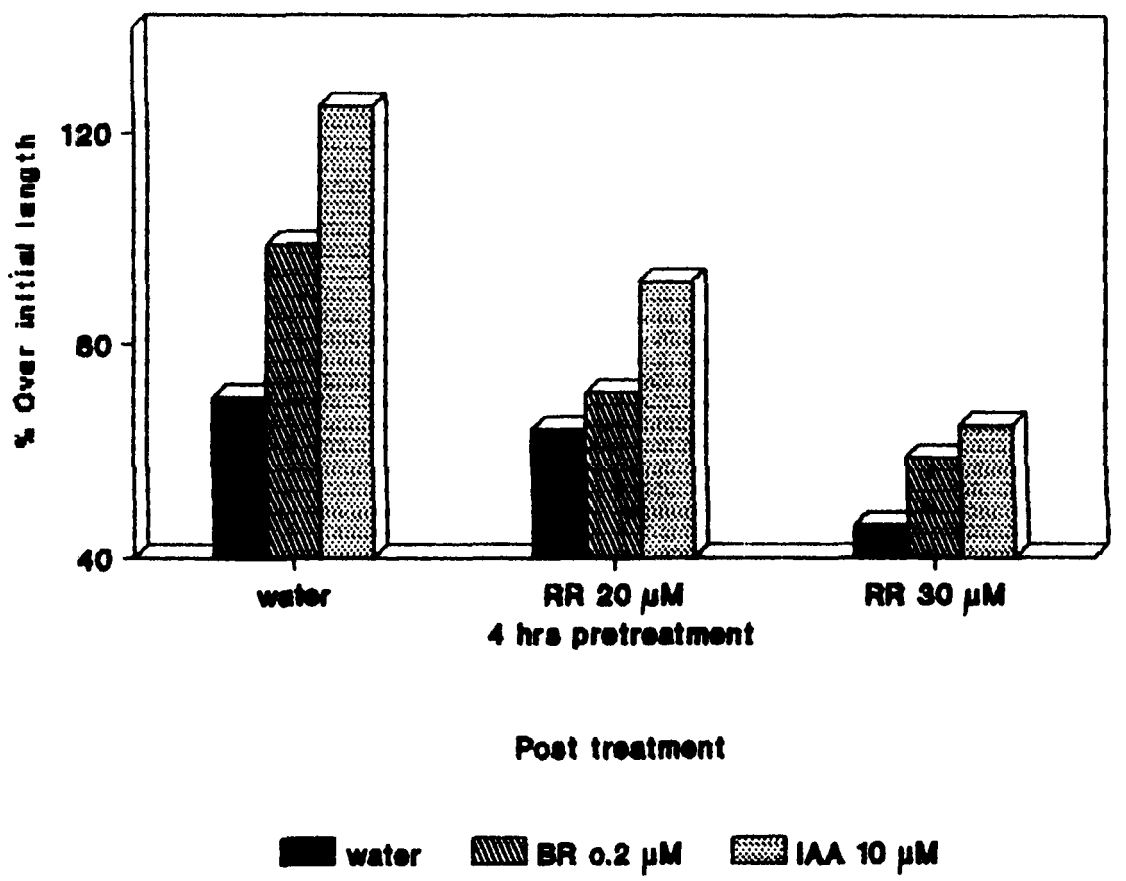


Fig. 34b: Influence of pretreatment of coleoptile segments with RR on IAA and BR induced growth.

[Coleoptile segments were pretreated with water or different concentrations of RR (2Ø and 3Ø µ M) for a period of 4 hours and then transferred to water or IAA (1Ø µ M) or BR (Ø.2 µ M) medium. Final length of segments were measured after 24 hours of post treatment

were incubated in a medium containing IAA and BR at different concentrations of Verapamil.

Coleoptile segments incubated over IAA or BR with 50 or 150 μM verapamil showed significant reduction in elongation growth (Fig. 35a). For instance, coleoptile segments over IAA and BR medium showed 97 and 78 per cent increased growth, respectively over the original length. In the presence of 1.0 μM verapamil along with IAA and BR, the increase in growth was only 24 and 23 per cent, respectively.

The influence of pretreatment of coleoptile segments with different concentrations of verapamil on IAA and BR induced elongation was examined (Fig. 35b). Coleoptile segments pretreated with 0.2 and 1.0 μM of verapamil for 4 hours showed significantly reduced growth in basal, IAA or BR medium. With increasing concentration of Verapamil used for pretreatment, further reduction in growth of coleoptile segments was noticed.

4.4.2 EXPERIMENT WITH CALCIUM ENRICHED TISSUE

To understand the role of calcium in IAA and BR induced elongation growth in wheat coleoptile tissue, the endogenous calcium concentration in the coleoptile was increased by germinating the seeds in Vermiculite medium irrigated with one per cent calcium chloride solution. Coleoptile segments with elevated calcium levels

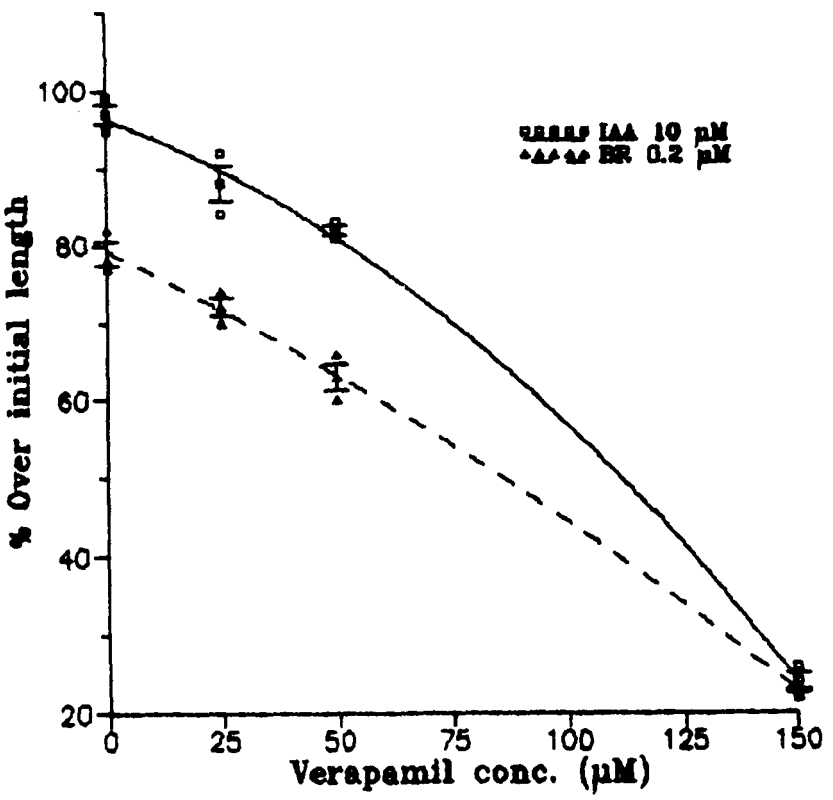


Fig. 35a: Effect of different concentrations of VERAPAMIL on IAA and BR induced growth in wheat coleoptile segments.

[Coleoptile segments were incubated in medium containing IAA (1Ø µ M) or BR (Ø.2 µ M) with different concentrations of verapamil. Final length of coleoptile segments were measured at the end of 24 hours incubation period.]

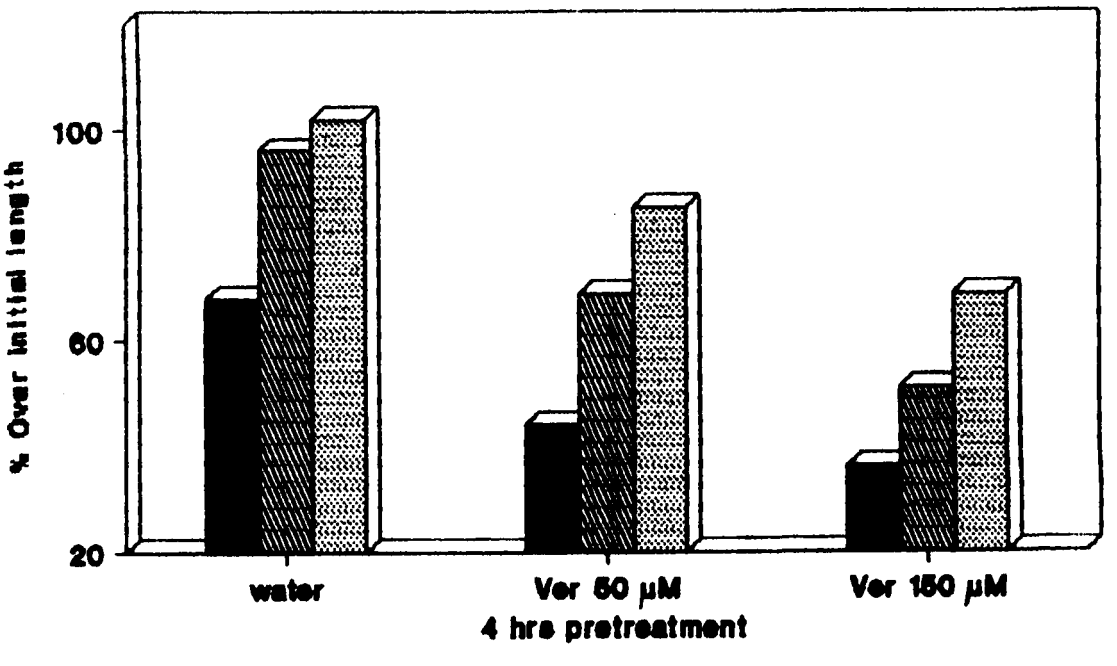


Fig. 35b: Influence of pretreatment of coleoptile segments with verapamil on IAA and BR induced growth.

[Coleoptile segments were pretreated with water or different concentrations of verapamil (50 and 150 µM) for a period of 4 hours and then transferred to water or IAA (10 µM) or BR (0.2 µM) medium. Final length of segments were measured after 24 hours of post treatment.]

were used to see the role of calcium on auxins and BR induced elongation growth.

1. Endogenous calcium concentration in the coleoptile tissue developed in calcium enriched medium:

The calcium concentration in the coleoptile tissue developed over calcium enriched medium was nearly 27 per cent more than the tissue developed over vermiculite medium irrigated with distilled water (Fig. 36).

2. Influence of IAA and BR on growth responses in coleoptiles with elevated calcium levels:

The influence of elevated calcium concentration in the tissue on IAA and BR induced elongation growth was examined. Coleoptile segments from the seedlings from normal and calcium enriched rooting medium were floated over basal medium, IAA (10 μM) and BR (0.2 μM). The final length of the coleoptile segments was measured after incubation for 24 hours in the medium.

Coleoptiles developed from the seedlings raised over calcium enriched medium showed significantly more elongation (Fig. 37) Coleoptile segments with normal level of calcium showed 60, 106 and 88 per cent increase in length over the original length in basal, IAA and BR medium, respectively. The corresponding values of coleoptile segments with elevated calcium concentration

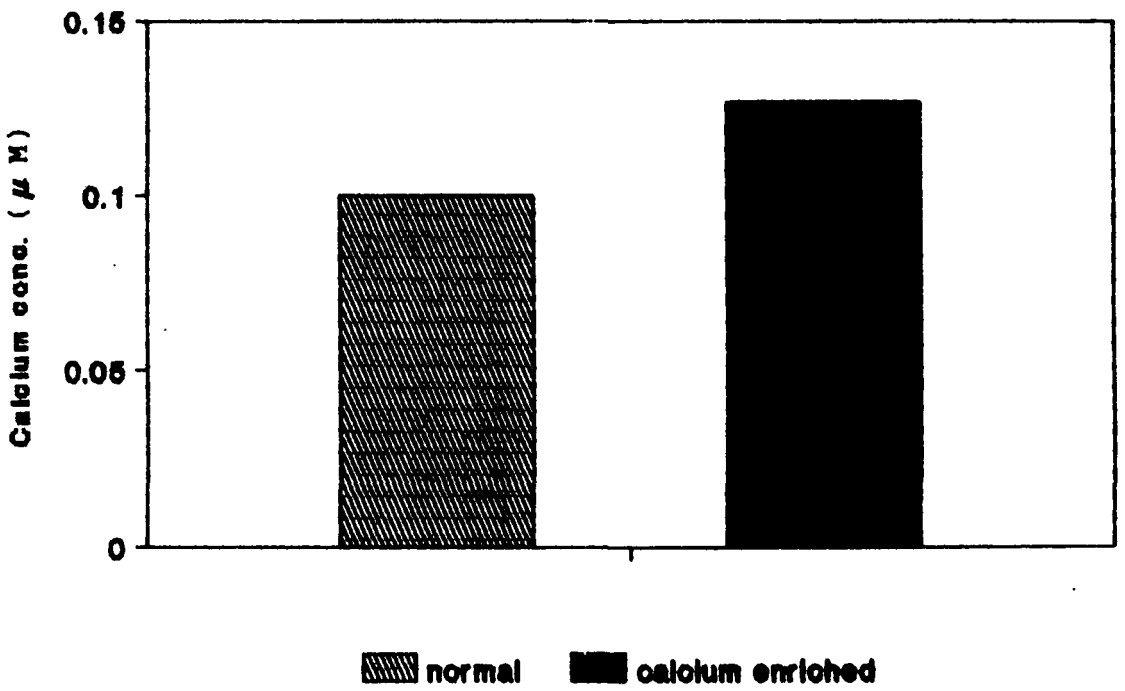


Fig. 36: Calcium concentration in normal and calcium enriched coleoptile tissue.

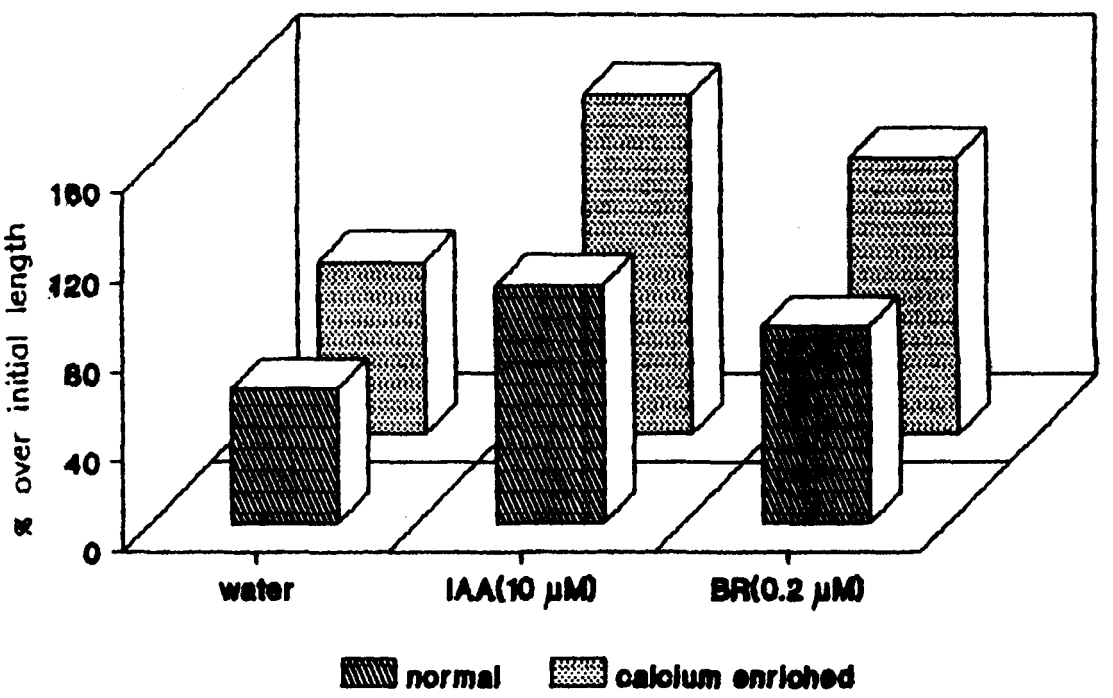


Fig. 37: Growth of normal and calcium enriched coleoptile segments in water, IAA, and BR media.

were 76, 151 and 123 per cent. This data clearly indicate that coleoptile segments with high calcium concentration showed more growth.

The role of calcium in IAA and BR induced elongation growth of coleoptile segments in calcium enriched tissues was examined by using different chemicals which reduced active calcium concentration in the cytosol.

4.4.2.1 EXPERIMENTS USING EGTA

The influence of EGTA on IAA and BR induced elongation in coleoptile tissue with normal and enriched calcium concentration was examined. Presence of EGTA (5 mM) inhibited both IAA and BR induced elongation in coleoptiles with normal and enriched calcium levels (Table 10). In the presence of EGTA, final length of coleoptiles were similar in all treatments.

4.4.2.2 EXPERIMENTS USING CPZ

The effect of calcium calmodulin inhibitor CPZ on IAA and BR induced elongation growth was studied in coleoptiles having normal and enriched level of calcium. Presence of CPZ (140 μM) significantly reduced the growth of coleoptiles in the basal medium as well as in IAA and BR.

CPZ was also effective in significantly reducing the growth of coleoptile with enriched calcium concentration (Table 11). Coleoptile with enriched calcium concentration

Table 10 : Effect of EGTA on IAA and BR induced elongation in wheat coleoptile segments with normal and elevated calcium levels.

(Final length of coleoptile segments in mm)

Treatments	Basal medium	IAA 10 µM	BR 0.2 µM
Coleoptile with normal Ca levels			
-EGTA	16.00 ± 0.13	19.43 ± 0.16	18.80 ± 0.15
+EGTA	14.92 ± 0.11	15.41 ± 0.13	15.39 ± 0.13
Coleoptile with elevated Ca levels			
-EGTA	17.67 ± 0.14	22.69 ± 0.20	21.37 ± 0.18
+EGTA	15.50 ± 0.13	15.70 ± 0.13	15.70 ± 0.13
CD at 5%	2.19		

Table 11 : Effect of CPZ on IAA and BR induced elongation in wheat coleoptile segments with normal and elevated calcium levels.

(Final length of coleoptile segments in mm)

Treatments	Basal medium	IAA 10 µM	BR 0.2 µM
Coleoptile with normal Ca level			
-CPZ	16.37 ± 0.13	19.66 ± 0.17	18.72 ± 0.15
+CPZ	14.70 ± 0.13	14.33 ± 0.11	14.73 ± 0.12
Coleoptile with elevated Ca level			
-CPZ	17.43 ± 0.14	22.10 ± 0.18	21.33 ± 0.17
+CPZ	12.53 ± 0.10	15.31 ± 0.12	15.57 ± 0.12
CD at 5%	2.97		

showed significantly more elongation in IAA and BR medium even in the presence of CPZ. For example, the final length of coleoptile with normal levels of calcium in IAA+CPZ and BR+CPZ treatments were 14.3 and 14.7 mm. At elevated calcium level in the corresponding treatment, the final lengths of coleoptiles were 15.3 and 15.6 mm, respectively.

4.4.2.3 EXPERIMENTS WITH RUTHENIUM RED

Ruthenium Red (20 mM) significantly reduced the growth of coleoptile at normal level of calcium (Table 12). The final length of the coleoptile segments was significantly less in the medium containing RR. Coleoptile segments with elevated calcium levels incubated in IAA+RR and BR+RR also showed significant inhibition of elongation compared to the growth in IAA or BR medium. Although RR was effective in significantly inhibiting the growth of coleoptile tissue in IAA and BR media, the inhibitory effect of RR was not significant in basal medium.

4.4.2.4 EXPERIMENTS USING VERAPAMIL

Verapamil also significantly reduced the growth of coleoptile segments in basal as well as in IAA and BR media (Table 13). The effect of verapamil in reducing coleoptile elongation was significant in coleoptile having normal or elevated calcium concentration. For

Table 12 : Effect of ruthenium red on IAA and BR induced elongation of wheat coleoptile segments with normal and elevated calcium levels.

	(Final length of coleoptile segments in mm)		
Treatments	**Basal medium**	**IAA 10 µM**	**BR 0.2 µM**
Coleoptile with normal Ca level			
-RUTHENIUM RED	16.23 ± 0.14	19.67 ± 0.17	18.80 ± 0.16
+RUTHENIUM RED	13.93 ± 0.11	16.60 ± 0.13	15.90 ± 0.12
Coleoptile with elevated Ca level			
-RUTHENIUM RED	17.31 ± 0.15	21.94 ± 0.18	21.30 ± 0.18
+RUTHENIUM RED	16.27 ± 0.14	17.66 ± 0.15	17.60 ± 0.16
CD at 5%	3.03		

Table 13 : Effect of verapamil on IAA and BR induced elongation of wheat coleoptile segments with normal and elevated calcium levels.

(Final length of coleoptile segments in mm)

Treatments	Basel medium	IAA 10 µM	BR 0.2 µM
Coleoptile with normal Ca level			
-VERAPAMIL	16.41 ± 0.13	19.12 ± 0.17	18.75 ± 0.17
+VERAPAMIL	12.84 ± 0.10	14.56 ± 0.12	13.80 ± 0.11
Coleoptile with elevated Ca level			
-VERAPAMIL	17.81 ± 0.14	22.08 ± 0.19	21.66 ± 0.18
+VERAPAMIL	15.13 ± 0.13	16.00 ± 0.14	15.54 ± 0.13
CD at 5%	3.20		

example, in coleoptile with normal level of calcium, the presence of verapamil in the medium reduced IAA induced growth from 19 mm to 15 mm. In coleoptiles with elevated calcium levels the corresponding reduction in elongation due to verapamil was from 22 mm to 16 mm.

Table 14 presents the summary of the results of the effect of EGTA, CPZ, RR and verapamil on reducing the IAA and BR induced growth in coleoptile segments having normal and elevated calcium levels. All the chemicals used were effective in reducing the growth of the coleoptile tissue in basal, IAA and BR. All the inhibitors showed a greater degree of growth inhibition in coleoptile with elevated levels of calcium. Amongst the chemicals used EGTA, CPZ and Verapamil showed higher extent of growth inhibition compared to RR.

These results clearly demonstrate that calcium concentration plays an important role in auxins and BR induced elongation growth of coleoptile tissue. In coleoptile tissue with elevated calcium concentration, IAA and BR induced greater degree of elongation growth. When the free calcium pool was reduced by sequestering with EGTA or when calcium entry is blocked by specific calcium channel blockers like lanthanum chloride, Verapamil and RR, there was significant reduction in IAA as well as BR induced growth. Calcium calmodulin

Table 14 : Effect of EGTA, CPZ, RR and VERAPAMIL on the extent of inhibition of growth on normal or calcium elevated wheat coleoptile segments.

(Per cent inhibition of growth over the respective control)

Chemical	Calcium level in the coleoptile	Basal medium	IAA 10 µM	BR 0.2 µM
EGTA	Normal	6.75	20.69	18.14
	Elevated	12.28	30.81	26.53
CPZ	Normal	10.20	27.10	21.31
	Elevated	28.11	30.72	27.00
RR	Normal	14.17	15.61	15.42
	Elevated	6.01	19.51	17.37
VERAPAMIL	Normal	21.75	23.85	26.40
	Elevated	15.05	27.54	27.79

inhibitor-CPZ was also effective in reducing IAA and BR induced elongation growth in coleoptile tissue.

4.4.3 ROLE OF ETHYLENE AND CALCIUM IN BR INDUCED LEAF LAMINA INCLINATION IN RICE SEEDLINGS

In the first part of the results it has been shown that BR induces leaf lamina inclination in intact seedlings and in excised leaf segments consisting of leaf sheath, ligule and lamina. Further it was also shown that none of the other hormones were effective in inducing leaf lamina bending.

Experiments were conducted to study the mode of action of BR in inducing leaf lamina inclination in intact rice seedlings.

4.4.3.1 INFLUENCE OF ETHYLENE INHIBITORS ON RICE LEAF LAMINA INCLINATION

Ethylene induces epinasty in many species. The influence of ethylene is more pronounced in seedlings grown in dark. In our experiments, the role of ethylene in BR induced leaf lamina inclination was studied by using two potent ethylene synthesis and ethylene action inhibitors. The second leaf lamina of eight day old rice seedlings were treated with water, Silver thiosulphate-STS (5 μM) and Cobalt chloride-$CoCl_2$ (500 μM). Twenty four hours after pretreatment, BR at a concentration of 0.02 μM was applied to leaf lamina and extent of inclination was measured at the end of 48 hours (Fig. 38

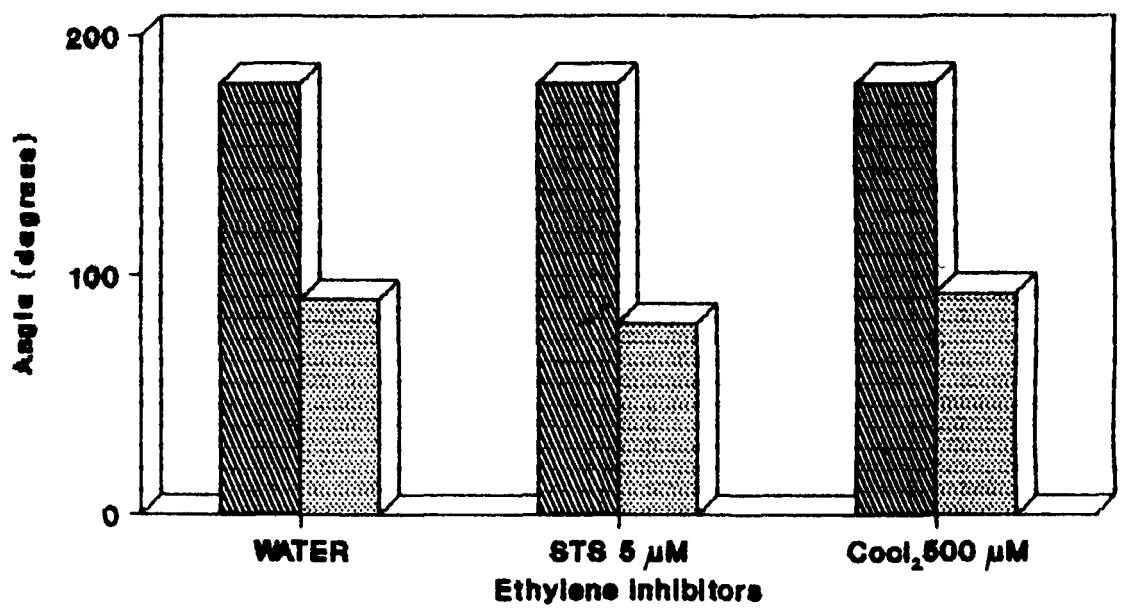


Fig. 38: Effect of ethylene inhibitors on BR induced leaf lamina inclination in rice sedlings.

(In intact rice seedlings, 1Ø μl of STS (5 μ M) or $CoCl_2$ (5ØØ μ M) was applied to the ligule of the second leaf. Twenty four hours after application of ethylene inhibitors, 1Ø μl of water or BR (Ø.Ø2 μ M) was applied to the same spot. Angle of bending was measured 48 hours after application of BR.)

and Plate 6). The results indicated that both ethylene synthesis inhibitor-Cobalt and Ethylene action inhibitor-Silver were not effective in overcoming BR induced leaf lamina bending. These results indicates that BR induced leaf lamina inclination is not due to BR induced increase in ethylene biosynthesis.

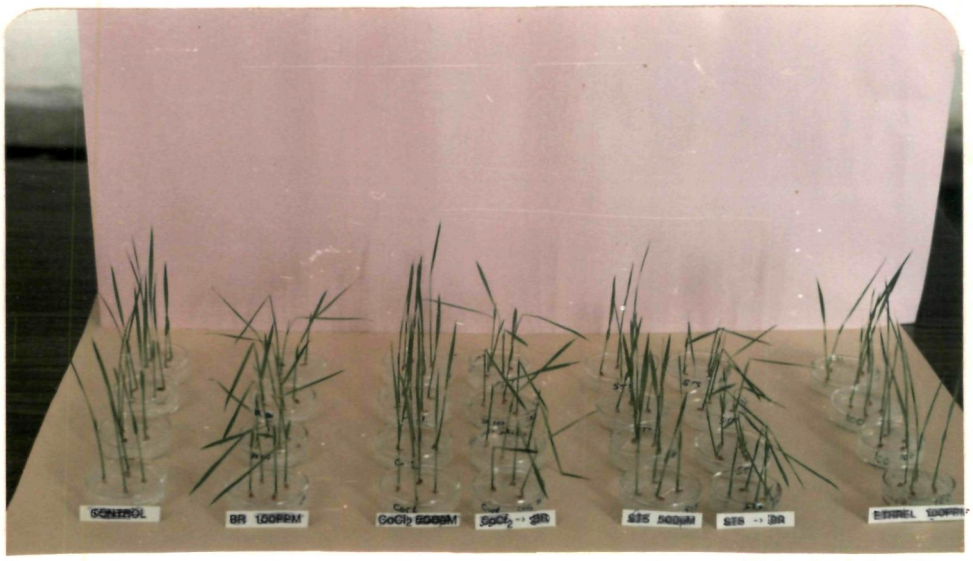
4.4.3.2 ROLE OF CALCIUM IN BR INDUCED LEAF LAMINA INCLINATION

The role of calcium in BR induced leaf lamina inclination was tested by using calcium sequestering agent, EGTA and calcium channel blockers, RR and verapamil.

EGTA pretreatment reduced the effect of leaf lamina inclination in rice seedlings. With increase in concentration of EGTA pretreatment, there was reduction in extent of bending (Table 15 and Plate 7).

Ruthenium red was also effective in reducing BR induced leaf lamina bending in intact rice seedlings (Plate 8). A concentration dependent reduction in bending was observed with increase in concentrations of RR. For example, the angle of bending in BR was 90. With 25 μM RR, the angle was 140 With further increase in concentration of RR 50 or 100 μM , the leaf lamina angle was 148 and 155

Pretreatment of leaf lamina with verapamil a calcium channel blocker, also reduced BR induced lamina



1 2 3 4 5 6 7

Plate 6: Influence of ethylene inhibitors $CoCl_2$ and STS on BR induced leaf lamina inclination in rice seedlings, cv. Tan-gin-bozu.

1) Control,
2) BR (Ø.Ø2 µM),
3) $CoCl_2$ (5ØØ µM),
4) $CoCl_2$ ---> BR,
5) STS (5 µM),
6) STS ---> BR, and
7) Ethrel (2ØØ µM)

Table 15 : Effect of calcium inhibitors on BR induced leaf lamina inclination (degrees) in Rice seedlings.

Treatments	water	BR (0.2 µM)
water	180	90
EGTA 2.5 mM	180	122
EGTA 5 mM	180	135
CD at 1 % 7.04		
RR 25 µM	180	140
RR 50 µM	180	148
RR 100 µM	180	153
CD at 1 % 1.817		
Ver 0.2 µM	180	129
Ver 0.6 µM	180	132
Ver 1.0 µM	180	156
CD at 1 % 0.806		

Plate 7: Pretreatment effect of EGTA on BR induced leaf lamina inclination of rice seedlings: 1) Control, 2) BR Ø.Ø2 μM, 3)EGTA 2.5 mM ---> BR, and 4) EGTA 5.Ø mM ---> BR.

Plate 8: Pretreatment effect of RR on BR induced leaf lamina inclination of rice seedlings: 1) Control, 2) BR Ø.Ø2 μM, 3) RR 2.5 μM ---> BR, and 4) RR 5.Ø μM ---> BR.

inclination and it was concentration dependent. With increase in concentration of verapamil, there was marked reduction in BR induced bending.

The results of these experiments indicates that there was an involvement of calcium in BR induced lamina inclination in intact rice seedlings.

4.5 INFLUENCE OF BR ON GROWTH AND PRODUCTIVITY

4.5.1 Effect of BR on seedling growth rates in horsegram

Internodal elongation: In this experiment the influence of BR on internodal elongation in young seedlings of horsegram was studied and related to the effect of other hormones like Gibberellins and cytokinins.

Horsegram plants were raised in small plastic bowls and plants were thinned to retain only eight plants per pot. When the plants were 10 days old the cotyledonary leaves of the plants were smeared with known amount of BR, BA and GA. The influence of growth regulator treatments on internodal length was determined on 6^{th} and 9^{th} days after application of BR, BA and GA as shown in Fig. 39a. Amongst the growth regulators used, GA was superior to BR and BA. Both at 1 and 10 ppm of GA concentration induced marked increase in internode elongation. BR particularly at a concentration of 10 ppm showed a remarkable increase in the internodal length. Cytokinin (BA) was not effective in increasing internode

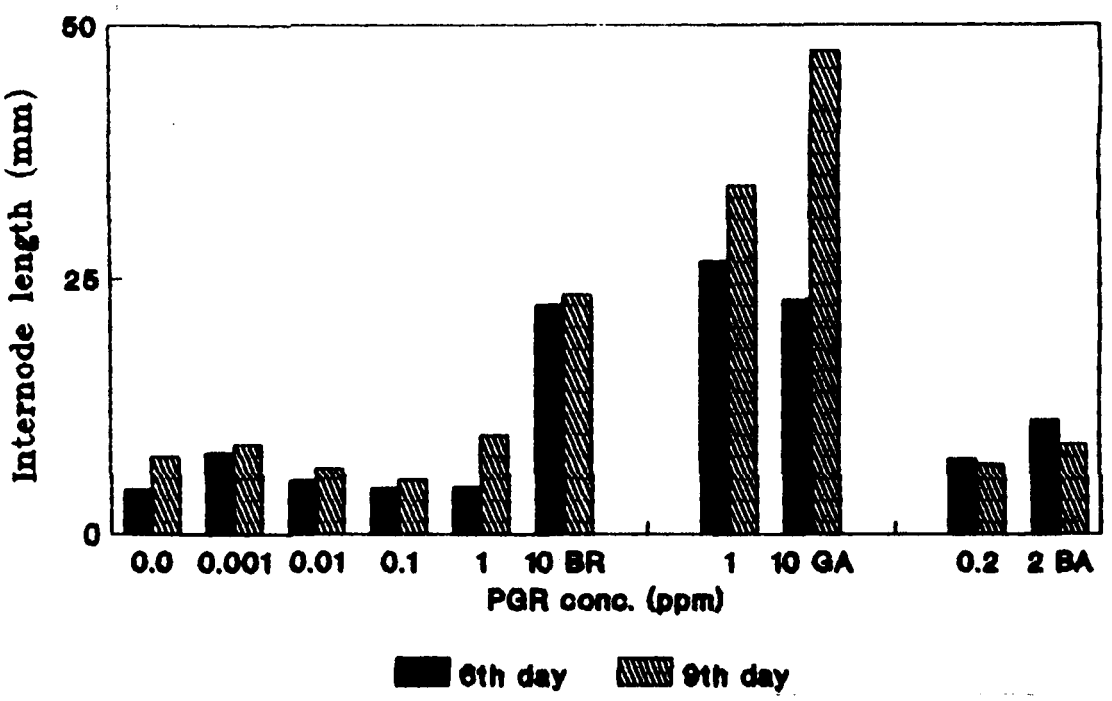


Fig. 39a: Influence of different concentrations of BR, GA, and BA on internode elongation in hores gram seedlings.

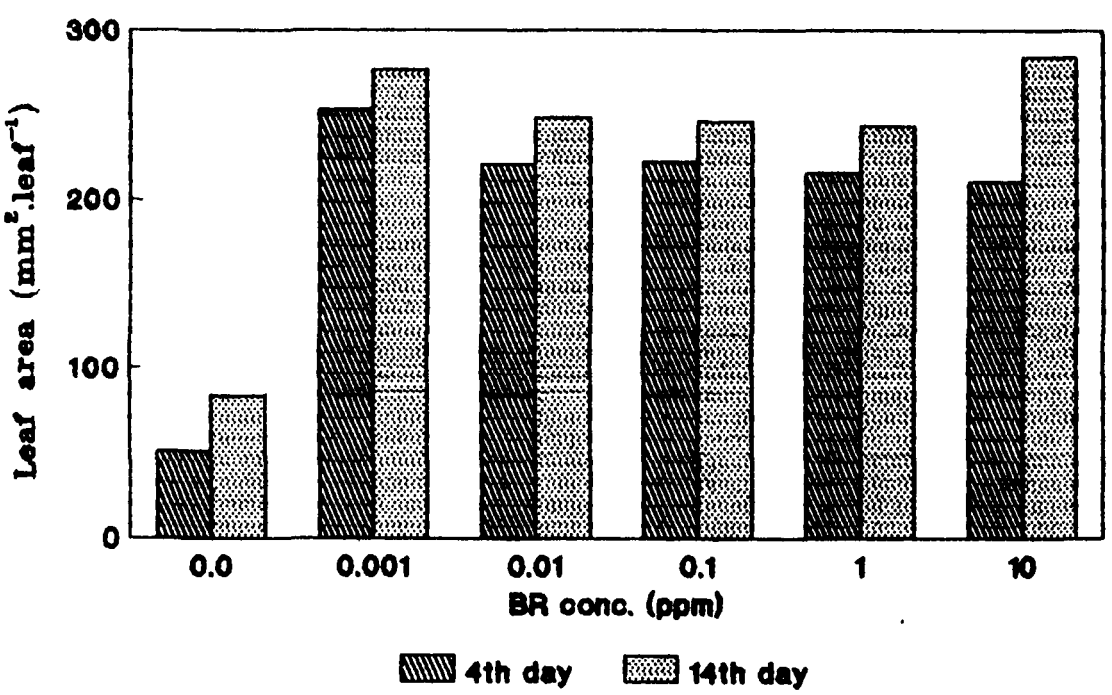


Fig. 39b: Effect of BR on leaf area of first trifoliate leaf ($mm^2.leaf^{-1}$) in horsegram.

elongation. Results of this experiment indicate that GA and BR, when applied to the seedling results in significant increase in internodal elongation.

Leaf area development: Experiments were conducted to study the effect of various concentrations of BR on leaf area expansion in horsegram seedlings. Leaf area of first trifoliate leaf was determined on 4^{th} and 14^{th} day after the application of BR as shown in Fig. 39b and Plate 9.

These results indicate that BR was effective in increasing leaf area. However, the differences between different concentrations of BR was not marked. On 4^{th} and 14^{th} day, there was 392 and 445 per cent increase respectively in leaf area at 0.001 ppm concentration of BR compared to control. Thus, BR was effective in increasing leaf area in young seedlings of horsegram.

4.5.2 EFFECT OF BR ON RICE LEAF LAMINA EXPANSION

In this set of experiments, the effect of BR and IAA and in combination of both on leaf lamina expansion was studied in rice seedlings. Table 16 shows the leaf expansion on application of growth regulators. A marked increase in expansion of the leaf was observed in the leaf which received BR, as well as, the new leaf which was produced above the treated leaf. Amongst the various treatments used, maximum leaf expansion was observed in the treatment where only BR was used to treat the leaf.

Photos Missing

Plate 9: Influence of BR on leaf area development in horse gram seedlings.

Table 16 : Effect of BR, IAA and BR+IAA on leaf expansion in rice leaf lamina inclination test.

Treatments	Leaf area(cm^2)	
	1st leaf	2nd leaf
Water	0.310	0.930
BR 10 ppm	0.315	1.310
IAA 10 ppm	0.300	1.150
BR + IAA	0.310	1.270
CD at 1 %	0.03	

4.5.3 EFFECT OF BR ON GROWTH AND PRODUCTIVITY IN GRAPES

In many test systems, BR induces cell elongation and also cell expansion. With an objective to use this property in increasing productivity, the influence of BR on berry growth in grapes was evaluated in three field experiments. Two field experiments were conducted on the variety Anab-E-Sahi and the other experiment on the variety Dilkush.

Experiment No. 1: Variety: Anab-E-Sahi

GA treatment to clusters significantly increased bunch weight (Table 17 and Plate 10). Immersing bunches in solution containing BR at concentration 0.001 and 5 ppm along with GA 25 ppm, increased the bunch weight further, compared to the treatment with GA alone. Maximum bunch weight was observed in this treatment where GA 25 ppm + BR 0.01 ppm were used. The increase in bunch weight in concentration of GA (25 ppm + BR 0.01ppm) was 21 per cent compared to untreated bunch and 7 per cent compared to treatment in which only GA 25 ppm was used.

Observations recorded on berry weight from different positions of the bunch indicated that the weight of berries at all positions of the bunch increased due to the treatment. Particularly berry in the bottom position of the bunches showed marked increase.

Table 17 : Effect of Brassinosteroid on productivity of grapes (Variety : ANAB-E-SAHI).

Treatments (ppm)	Bunch weight (g)	Berry No. per bunch	Berry weight (g).5 berries^{-1}
Control	687.5	100	7.55
0.001 BR	692.5	119	7.90
0.01 BR	695.0	105	8.30
0.1 BR	705.0	110	8.37
1.0 BR	740.0	119	8.00
5 BR	687.0	109	8.40
25 GA	787.0	114	7.30
25 GA + 0.001 BR	770.0	102	9.30
25 GA + 0.01 BR	840.0	117	7.60
25 GA + 0.1 BR	815.0	119	7.90
25 GA + 1.0 BR	795.0	115	7.10
25 GA + 5.0 BR	832.5	128	7.80
CD at 5 %	85.54	-	-

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Plate 1Ø: Effect of BR and GA on bunch growth of grapes cv. Anab-E-sahi.

Quality parameters like acid content, sugar content and total soluble solids in the berries did not differ significantly between the treatments.

Experiment No. 2: Variety: Anab-E-Sahi

In the second field experiment also significant increase in bunch weight was recorded when BR or GA was used (Table 18). Even BR treatment alone was effective in increasing the bunch weight compared to the untreated bunches. However, Gibberellin treatment was more effective than BR 0.1 ppm and 1 ppm alone. BR along with gibberellin was highly effective in increasing bunch weight more than that of gibberellin treatment. The per cent increase in bunch weight at the most effect combination treatment of 25 ppm GA + 1.0 ppm BR worked out to 15 per cent compared to untreated bunches and 8 per cent compared to GA treatment alone.

Experiment No. 3: Variety: Dilkush

The effect of BR and GA alone or the combination treatment effect on yield and quality parameters were tested in the cv. Dilkush. All these are listed in Table 19 and Plates 11-14.

Bunch weight at harvest was significantly more in one and five ppm BR and GA treatment. A linear increase in bunch weight was noticed with increase in concentration

Table 18 : Effect of Brassinolide on productivity of grapes (Variety : Anab-E-Sahi).

Treatment (ppm)	Bunch weight (g)
Control	782
0.1 BR	822
1.0 BR	822
25 GA	899
25 GA + 0.1 BR	949
25 GA + 1.0 BR	972
CD at 1 %	29.57

Table 19 : Effect of BR, GA and BR + GA combination treatement on bunch weight an berry characteristics in grapes(Variety:DILkUSH)

Treatments (ppm)	Weight/ branch (g)	Weight of five berries			Mean berry weight	No.of berries/ bunch	Volume (ml)			Length of 5 berries(cm)			TSS(%)		
		Top	Middle	Bottom			Top	Middle	Bottom	Top	Middle	Bottom	Top	Middle	Bottom
Control	746	34	43	38	38	94	31	40	34	18	20	19	13	14	14
0.1 BR	752	40	47	41	43	84	38	44	38	19	21	19	13	16	15
1 BR	777	40	44	35	40	90	34	42	34	19	22	18	14	15	14
5 BR	796	38	46	39	40	89	36	44	38	19	21	19	14	14	13
25 GA	908	44	50	45	46	96	46	49	42	22	23	21	13	13	13
0.1 BR + 25 GA	926	44	51	42	45	90	43	49	43	22	24	21	13	13	13
1 BR + 25 GA	940	44	54	46	46	88	43	50	44	22	23	21	14	14	13
5 BR + 25 GA	1043	45	54	43	47	112	47	50	43	23	25	22	13	13	14
CD at 1 %	30.5	2.9	2.8	3.0	1.6	-	4.8	3.1	3.5	1.2	0.8	1.2	1.1	0.5	0.8

of BR used for dipping the bunches. GA was highly effective in increasing bunch weight when compared to different concentration of BR. Bunch weight increased further when GA + BR combination treatment was used. The bunch weight was maximum when BR 5 ppm + GA 25 ppm was used for dipped bunches. In the most effective combination treatment of BR (5 ppm) + GA (25 ppm), the increase in bunch weight was 40 and 15 per cent compared to untreated bunches and GA 25ppm treatment alone, respectively.

Data on individual berry weight at different positions of the bunch indicated that BR and GA and their combinations were effective in increasing the berry weight significantly, particularly in top and middle portion of the bunch. Treatment of bunches with Gibberellic acid alone or in combination with various concentration of BR also resulted in significant increase in berry weight from bottom position of the bunch also. The data on mean berry weight clearly indicated that both BR and GA are effective in increasing the berry weight significantly.

Number of berries per bunch varied from 84 to 112. However, the differences were not statistically significant.

Volume of the individual berries from different positions of the bunch also showed significant increase in most of the treatment. In all the positions of the bunches GA treatment alone or GA in combination with various concentration of BR, increased the volume of the individual berries. Similarly, the length of the berries from different position of the bunches, also showed a marked increase in GA with BR treatment.

The thickness of bunch skeleton was more in GA and BR combination treatment compared to GA alone, which is more than control, as shown in Plate 15.

4.5.4 EFFECT OF BR ON GROWTH AND PRODUCTIVITY IN SUNFLOWER

The influence of foliar applied BR on growth and productivity of sunflower crop was studied in two field experiments during summer season. The crop was raised under irrigated condition and all the package of practices recommended for the crop was followed.

Experiment 1: The sunflower crop was harvested on 95th day after sowing and the weight of seeds produced in an area of one square meter land recorded and seed yield per hectare land area was calculated (Table 20). These results indicated that yield of sunflower seeds increase with increasing concentrations of BR applied as foliar spray. The maximum seed yield was with 5 ppm foliar application of BR. Maximum increase in productivity to an

Photo Missing

Plate 15: Effect of GA and GA with BR on bunch rachis morphology of grapes cv. Dilkush.

Table 20 : Effect of brassinolide on sunflower yield.

Treatments	days after sowing						
	20	40	60	20+40	20+60	40+60	20+40+60
	Seed (g.earhead^{-1})						
1 ppm	26.0	27.2	26.4	28.4	28.9	27.3	29.0
2 ppm	26.3	26.5	25.9	28.6	27.9	28.6	29.6
5 ppm	27.0	27.5	25.4	29.7	28.1	29.1	30.6
10 ppm	25.9	26.3	26.8	28.2	28.7	28.1	28.5
control	25.1						
CD at 5%	For BR concentrations (A)			2.12			
	For days after sowing (B)			2.51			
	Interactions (A X B)			5.62			
	Seed (g.sq mt^{-1})						
1 ppm	142	154	153	153	154	157	162
2 ppm	151	150	150	154	155	156	160
5 ppm	155	152	146	157	157	159	165
10 ppm	149	147	150	151	149	153	158
control	143						
	Seed (q.ha^{-1})						
1 ppm	13.9	15.1	15.0	15.0	15.1	15.4	15.9
2 ppm	14.8	14.7	14.7	15.1	15.2	15.3	15.7
5 ppm	15.2	14.9	14.3	15.4	15.4	15.6	16.2
10 ppm	14.6	14.4	14.7	14.8	14.6	15.0	15.5
control	14.0						

extent of 15 per cent over control, was recorded for the treatment in which BR was applied at a concentration of 5 ppm at three stages of plant growth namely, 20+40+60 days after sowing (DAS). However, the differences between the treatments were not statistically significant.

Experiment 2: This experiment was conducted in summer season under irrigated condition. Foliar spray of BR was given on 30 and 40^{th} day after germination and observations on biomass accumulated in various organs of the plant, and seed yield were recorded and also listed in Table 21.

Observations recorded at harvest indicated that foliar application of one ppm BR resulted in marginal increase in plant height and total biomass produced by the plant. Seed yield was relatively more in plants treated with different concentrations of BR. Maximum seed yield was recorded with one ppm BR. However, the differences between the treatments in seed yield were statistically insignificant.

Table 21 : Effect of Brassinolide on biomass produced by the plant and seed yield in sunflower.

Treatment	Plant hight at harvest (cm)	Leaf wt. $g.plant^{-1}$	stem wt. $g.plant^{-1}$	Empty thalamus wt. $g.plant^{-1}$	Seed wt. $g.plant^{-1}$	Total biomass $g.plant^{-1}$
Control	108	10.30	25.06	9.30	35.7	80.13
Foliar spray 1 ppm BR	119	12.97	28.76	11.40	39.7	92.83
Foliar spray 2 ppm BR	115	12.64	24.84	10.71	36.5	84.69
Foliar spray 5 ppm BR	114	13.73	27.27	10.70	36.3	88.00
Foliar spray 10 ppm BR	117	12.60	26.37	11.10	35.6	85.67
CD at 5%	8.2	1.28	3.14	NS	NS	4.06

DISCUSSION

V DISCUSSION

Brassinosteroids are endogenously synthesized steroidal lactones, now shown to be present in a wide range of plant species. Yokota et al.(1988) reported the occurrence of over thirty different types of chemicals under group brassinosteroids. These chemicals are detected in various parts of the plant such as pollen, leaves, flowers, seeds, galls and stem.

Extensive studies on the biological activity of the chemical indicated that, these chemicals are responsive in a few bioassays standardized either for auxins or gibberellins or cytokinins. In a few specific bioassays, BR was as effective as or even more effective than the hormone for which the bioassay is standardized (Mandava et al., 1981; Yopp et al., 1981). This suggests the need for studying the relative effectiveness of BR in different test systems and also the need for developing a sensitive and specific bioassay for BRs. The BRs endogenously must be acting along with other natural hormones found in plants. The interaction of BR with other hormones, particularly with auxins, which also induces cell elongation is another important area which needs further experimentation. The mode of action of BR in inducing cell elongation and finally the agricultural use of these

chemicals need further experimentation. With all this information in the background experiments were conducted with the following objectives.

1. To identify the relative effectiveness of BR and auxins on growth processes in a few test systems.
2. To quantify the interaction effect of BR with auxins on growth in a few test systems.
3. To know the mode of action of BR in promoting growth in test system, and
4. To know the effectiveness of BR in increasing growth and productivity in grapes and sunflower.

INFLUENCE OF BR ON GROWTH RESPONSES IN TEST SYSTEMS

Brassinosteroids were shown to promote cell division and cell elongation resulting in both an enhancement and acceleration of growth in many plant systems (Mitchell et al., 1970; Worley and Mitchell, 1971; Gregory, 1981). Many workers have reported that BR has similar effect as that of auxins in many test systems (Yopp et al., 1981; Mandava et al., 1981). In the present study experiments were designed to characterize the nature and range of plant growth promoting activity of BR using a few test system.

The results showed that BR elicited growth responses similar to that of an auxin in many test systems like wheat coleoptile segment elongation test and soybean stem section elongation test (Table 22).

Table 22 : Comparative effect of BR and other hormones in various test systems in the present study.

Test		Concentration range for linear response	Optimum concentration for maximum response	Extent of response
Wheat coleoptile segments	BR	0.002 to 20 µM	0.2 µM	36 % over control
elongation test	IAA	0.01 to 10 µM	10 µM	45 % over control
Maize mesocotyl segments	BR	0.002 to 40 µM	20 µM	86 % over control
elongation test	IAA	0.01 to 1 mM	10 µM	162 % over control
Soybean stem segment	BR	0.002 to 40 µM	0.2 µM	160 % over control
elongation test	IAA	0.01 to 1 mM	1 mM	100 % over control
Mung bean seedling growth	BR	0.002 to 40 µM	40 µM	
bioassay:				
Inhibition of hypocotyl length				90 % less than control
Inhibition of root elongation				90 % less than control
Radial expansion of the stem				195 % over control
Coleus petiole explant curvature test	BR	0.002 to 40 µM	40 µM	89 % reduction over control
Mung bean epicotyl elongation	BR	0.0002 to 20 µM	20 µM	275 % over control
test	GA	0.01 to 10 mM	10 mM	367 % over control
Tobacco leaf disc expansion	BR	0.02 to 2 µM	2 µM	
test:				
Fresh weight				10 % over control
Diameter				57 % over control
Rice leaf lamina inclination test:				
Excised leaf segments	BR	0.02 to 2 µM	2 µM	83 % less than control
Intact leaves	BR	0.02 to 0.1 µM	0.1 µM	83 % less than control
Cucumber cotyledon expansion	BR	0.002 to 20 µM	20 µM	19 % over control
test	BA	0.003 to 6.5 µM	6.5 µM	162 % over control
Leucina leucocephala	BR	0.2 µM		
pollen germination test:				
pollen Germination				32 % more than control
pollen tube length				70 % more than control in 30 minutes

The optimum concentration of BR required to induce maximum response in wheat coleoptile segment elongation test and also in soybean stem segment elongation test were only one fiftieth part, and over five thousandth part of IAA respectively indicating that BR is highly effective in inducing growth responses at very low concentration compared to that of auxins. In maize mesocotyl segment elongation test, the optimum concentration of BR required to induce maximum elongation was twice that of IAA.

Comparison of the magnitude of responses elicited by BR and IAA indicate that wheat coleoptile segments as well as soybean stem segments were more responsive to auxin than to BR. In both the test systems IAA was significantly superior to BR. The maize mesocotyl segments were, however, much more responsive to BR than to IAA. Similar study conducted earlier by Yopp et al. (1981) indicated that Brassinolide induces responses similar to IAA in a number of bioassays like bean hypocotyl hook opening, elongation of maize mesocotyl, pea epicotyl and azuki bean epicotyl and fresh weight increase in Jerusalem artichoke tuber section and in pea epicotyl sections. In azuki bean and dwarf pea epicotyl bioassays BR was found to induce greater response than that of IAA.

The elongation response of mung bean epicotyl tissue to BR was studied and compared with that of gibberellins. Similarly the influence of BR and cytokinin (BA) in

inducing expansion growth of excised cucumber cotyledons were compared.

In mung bean epicotyl elongation test, the optimum concentration of BR required to induce maximum response was two times that of gibberellic acid. At an optimum concentration, gibberellic acid enhanced the elongation to an extent of 367 per cent over control, compared to 275 per cent in case of BR, suggesting that mung bean epicotyl tissue is more sensitive to GA than to BR.

BR elicited a poor response in classical cytokinin bioassay in which cucumber cotyledon expansion was studied. BR at a concentration of 2 μM resulted in only 10 per cent increase in fresh weight compared to 160 per cent by benzyladenine.

To study the range of effectiveness of BR, its effect on a few other test systems were also assessed. BR was highly effective in coleus petiole explant curvature test in which the angle of curvature between the petioles located opposite to each other is reduced by BR. The effective concentration range was between 0.002 μM to 40 μM and a maximum reduction to an extent of 89 per cent was observed at the highest concentration of BR used in the test.

Tobacco leaf discs were used to study the influence of BR in inducing cell expansion. In this test system, BR

was effective in inducing expansion of leaf discs as well as in increasing the fresh weight of the leaf discs. At the optimum concentration of BR, there was 18 per cent increase in diameter of the leaf disc and 57 per cent increase in fresh weight of the discs indicating that BR induces cell expansion as well as water uptake by the tissue.

In a large number of test systems employed, responses similar to that of IAA was elicited by BR. BR was also active in gibberellin bioassay with etiolated bean hypocotyl and to a very less extent in cytokinin bioassay with excised cucumber cotyledons (Table 22). However, none of these bioassays were highly sensitive to BR.

With an objective of developing specific and sensitive bioassay for BR, different test systems were developed and standardized by different scientific groups. For the discovery of brassins and subsequent isolation and characterization of the active compound brassinolide, a bean second node bioassay was developed by Mitchell and Livingston (1968). Later Gregory and Mandava (1982) standardized a sensitive mung bean epicotyl bioassay. However,this bioassay was also found to be sensitive to Gibberellins. Wada et al. (1985) standardized wheat leaf unrolling test to identify brassinosteroids. In this test brassinosteroids showed activities opposite to that of auxins, which helped in

identifying BR from that of auxin. However, zeatine was also found to elicit similar response as that of BR in wheat leaf unrolling test (Wada et al., 1985).

Excised rice leaf lamina inclination test was standardized and found highly sensitive test for brassinosteroids by Wada et al. (1981). These workers also showed that brassinolide has extremely high activity in this test. In a later work, Wada et al. (1984) showed that IAA also showed similar activity in this bioassay. However, the lowest effective concentration of IAA required to induce measurable effect is about five orders of magnitude greater than BR. This test is often being used to identify brassinosteroids (Yokotaet al., 1982; Takatsuto et al., 1982).

In our experiments, in dwarf rice cv. Tan-gin-bozu, BR is highly effective in inducing leaf lamina inclination both in intact seedlings as well as in excised leaf segments. In intact young seedlings none of the hormones other than BR was effective in inducing the response. In some test systems, BR was shown to induce ethylene biosynthesis (Yopp et al., 1979; Yopp et al., 1981). Since ethylene is known to induce epinasty (Bradford and Yong, 1980), whether BR induced leaf lamina inclination is also mediated by ethylene biosynthesis was examined. Pretreatment of rice seedlings with ethylene biosynthesis inhibitor-cobalt chloride was not effective in inhibiting

BR induced leaf lamina inclination. Similarly application of ethylene action inhibitor-silver ion in the form of silver thiosulphate immediately after application of BR was also not effective in overcoming the BR induced rice leaf lamina inclination. This information indicates that BR induced leaf lamina inclination in dwarf rice Tan-gin-bozu is specific to BR and none of the other hormones was effective in inducing similar response.

From the results of the experiment conducted to study the comparative effects of BR and other hormones in a number of test systems, the following points emerge,

a) BR showed responses similar to IAA in wheat coleoptile segments elongation test. The optimum concentration of BR required to induce maximum elongation was very less compared to IAA.

b) BR induced elongation of soybean stem segment similar to that of IAA. The optimum concentration of BR required to elicit maximum response as well as the magnitude of response elicited by BR was significantly more compared to IAA.

c) BR was also effective in inducing growth responses in a wide range of other test systems. Example: Coleus petiole explant curvature, tobacco leaf disc expansion test and pollen germination in Leucina leucocephala.

d) BR elicited growth responses similar to that of GA in mung bean epicotyl segments test. The magnitude of response induced by BR is less compared to GA. However, in the cytokinin bioassay of excised cucumber cotyledon expansion, BR elicited poor response.

e) In dwarf rice cv. Tan-gin-bozu, BR induced leaf lamina inclination in both excised leaf segments and in intact leaves. In intact leaves, none of the other hormones was effective in inducing this response.

Dwarf rice cv. Tan-gin-bozu leaf lamina inclination in intact seedlings can be used as a simple, sensitive and specific bioassay for BR.

INTERACTION OF BR WITH AUXINS AND OTHER HORMONES IN A FEW TEST SYSTEMS

The interaction effect of BR with auxins on growth responses were tested in wheat coleoptile elongation growth, rice leaf lamina inclination, and expansion growth of isolated tobacco leaf discs on tissue culture medium.

In wheat coleoptile segments, presence of both IAA and BR together in the medium resulted in elongation growth more than that of either BR or IAA alone. This suggests that there is a marked interaction effect between IAA and BR in eliciting higher growth rates in coleoptile segments. Further, coleoptile segments pretreated with BR for three hours, and then transferred to IAA for 24 hours

post treatment, showed more growth compared to the growth induced by IAA or BR alone for 24 hours. However, after pretreatment with BR for 3 hours, washing the coleoptile segments with water before transferring to IAA medium significantly reduced the pretreatment effect of BR.It indicates that the effect is reversible and the binding of BR molecules to the action site might be loose.

Interestingly instead of BR pretreatment, if coleoptile segments were pretreated with IAA and then transferred to BR for posttreatment, the final growth was not more than that of IAA alone. This indicates that only pretreatment of tissue with BR or when BR is present along with IAA from the beginning of the treatment alone will help in increasing growth rates.

In dwarf rice leaf lamina inclination test BR is highly active in inducing leaf lamina bending at very low concentrations. Auxin was not effective in eliciting the bending responses. However, when BR and IAA were applied together, the extent of leaf lamina bending was more than that of BR alone. This data is indicative of interaction effect of BR and IAA in leaf lamina bending also.

In tobacco leaf disc expansion test, in the presence of NAA and BR together, a synergistic interaction was observed resulting in significant increase in fresh weight as well as increased expansion of leaf discs.

The results from all the three tests indicated a marked interaction effect between IAA and BR in inducing growth responses. Experiments conducted to study the effect of BR in inducing cucumber cotyledon expansion indicated that BR is not effective in inducing expansion growth when present in the medium. Significant interaction effect was also not observed between cytokinin and BR in cucumber cotyledon expansion bioassay.

A number of earlier reports also indicated a synergistic interaction between brassinosteroids and auxin in young elongating stem segments (Yopp et al., 1981; Katsumi, 1985 and Sasse, 1987, 1989). Single cell of carrots were also shown to respond synergistically to auxin and brassinosteroids in the culture media resulting in enhanced cell elongation (Sala and Sala, 1985). Similarly, auxin and brassinosteroids were shown to have synergistic interaction in bending responses of many tissues(Yopp et al., 1981; Cohen and Meudt 1983 and Meudt 1987). Conversely, Cerana et al. (1983a) reported that IAA and brassinosteroids act in an additive manner in azuki epicotyl section elongation. Many of these interaction effects suggest that brassinosteroid may be acting as a modulator of auxin action and Katsumi (1985) viewed that BR seems to be involved in some process of auxin action, enhancing it or sensitizing the tissue to the auxin action.

Many others feel that the action of brassinosteroids is independent to that of IAA. Sala and Sala (1985) showed that BR can induce elongation in auxin starved carrot cells. In etiolated wheat leaves, auxin and BR were shown to have opposite effects (Wada et al., 1985; Sasse 1989). Auxin was shown to inhibit the unrolling response of wheat leaves, whereas, BR promoted the unrolling. Romani et al. (1983) also reported that brassinosteroids and auxins have opposite effect in maize root segments. IAA was usually inhibitory to root growth whereas, brassinosteroids showed stimulatory effect. However, since root growth is stimulated by low concentration of endogenous auxin, external application of BR would have interacted with endogenous auxin to stimulate growth. Similarly, BR effect in inducing elongation growth of auxin depleted carrot cells also may be due to over sensitization of tissue to IAA in the presence of BR.

The interaction effect of auxin and BR in inducing the growth responses in plant tissues can be explained in three ways:

1) Auxin and BR might be having independent effect and in the presence of both the hormones, the response is more than that of any of the compounds alone.

2) BR may be increasing the endogenous free auxin concentration near the site of action resulting in

enhanced response to auxin in the presence of BR (Katsumi, 1985), and

3) BR might be sensitizing the tissue to respond more to auxin probably by increasing the affinity of receptor proteins to auxin (Katsumi, 1985).

MODE OF ACTION OF BR IN ELONGATION RESPONSES

Experiments were conducted to study the difference in the pattern of growth of coleoptile monitored at various intervals after incubating tissue in the medium of IAA or BR. Appreciable increase in IAA induced growth was observed from starting from 30 minutes after introducing IAA to the medium. BR induced elongation was observed only after a lag phase of 2 hours, indicating that physiological and biochemical changes leading to increased growth rate achieved early in IAA treated tissue. This rapid response of coleoptile segments to IAA is explained as acid induced growth by Rayle and Cleland (1977 and 1992).

H^+ EXTRUSION AND ACIDIFICATION OF THE MEDIUM

Auxin induced rapid elongation growth in excised stem segments is explained by "Acid growth theory" (Rayle and Cleland, 1977 and 1992). It was also shown that tissue incubated in auxin medium secretes acid within a

relatively short period of time and acidifies the medium (Rayle and Cleland, 1970 and 1977).

In the present investigation, coleoptile segments floated over auxin or BR medium also showed proton extrusion resulting in acidification of the medium. In coleoptile segments floated over auxin solution, there was relatively rapid increase in growth with concomitant reduction in the pH of the medium. Coleoptile segments floated over BR medium showed relatively late response in growth and also measurable reduction of pH was noticed relatively late.

When coleoptile segments were floated over media containing both IAA and BR, the initial growth response was similar to that of auxins. However, one hour after the beginning of incubation in auxin+ BR the elongation growth was more than that of auxin induced growth, similar trend was observed in acidification of the medium. These data also indicated that H^+ secretion can be casually related to auxin and BR induced elongation. These observations also emphasizes the need for an initial lag phase for BR induced growth.

In the presence of both IAA and BR in the medium, the growth of the coleoptile segments and extent of acidification of the medium was higher indicating an additive effect. Further, experimental results using DCCD

(Katsumi,1985) - an inhibitor of membrane bound ATPase also indicated that both IAA and BR induced elongation response needs activity of ATPase proton pump on membranes. These results tempt to conclude that both IAA and BR induce plasma membrane bound ATPase proton pump resulting in excretion of proton into cell wall at an enhanced rate, resulting in a decrease in apoplastic pH. The lowered wall pH then activates wall loosening process resulting in cell elongation. Experimental results from IAA and BR induced acidification of the medium and the influence of DCCD on IAA and BR induced growth suggests that,

1) Both IAA and BR induces proton excretion and thus acidification of the medium. A close relationship exists between extent of acidification and elongation response of the tissue, and

2) Inhibitors of membrane bound ATPase (DCCD) inhibit both IAA and BR induced growth.

INFLUENCE OF GALACTOSE ON IAA AND BR STIMULATED ELONGATION OF COLEOPTILE SEGMENTS

Galactose inhibits auxin induced growth of coleoptile segments after a lag period of 1 to 2 hours (Ordin and Bonner, 1957; Yamamoto et al., 1981; Yamamoto and Masuda, 1984), does not inhibit acid induced growth (Yamamoto et al., 1981). Galactose was shown to inhibit

formation of UDP-Glucose (Inouhe et al., 1986) and the incorporation of glucose into cell wall. (Cheng and Cleland, 1991; Yamamoto and Masuda, 1984). Further, Cheng and Cleland (1991) showed that galactose inhibits incorporation of glucose into both non-cellulosic and cellulosic components of the cell wall.

Galactose is not effective in inhibiting acid induced growth. Fusicoccin (FC) induced elongation, which occurs in response to FC induced cell wall acidification, was unaffected by galactose. Similarly, cell wall extension in response to exogenous acidic solution was also unaffected by galactose (Cheng and Cleland, 1991).

However, in the present study, galactose was effective in inhibiting both IAA and BR stimulated elongation of coleoptile segments. Addition of various amounts of galactose from 10 to 50 mM concentration progressively decreases both IAA and BR stimulated elongation of coleoptile segments more than 45 and 62 per cent at 50 mM concentration of galactose (Fig. 21a). Similarly, four hour pretreatment of coleoptile segments with 10 mM galactose also blocked the effect of IAA, BR. This piece of evidence indicates that like IAA and BR induced growth also involves synthesis of new cell wall material. BR may also induce incorporation of glucose to cell wall components and thus new cell wall synthesis is necessary for maintaining sustained elongation for a long period.

REQUIREMENT OF RNA AND PROTEIN SYNTHESIS FOR IAA AND BR INDUCED ELONGATION OF COLEOPTILE SEGMENTS

Auxin induced cell elongation sustained for a longer period, requires a continuous synthesis of RNA and proteins (Key et al., 1967; Moore, 1989; Muir, 1974). Recently, Breviario et al. (1992) by using specific inhibitors such as actinomycin D, 5-fluorouracil and cycloheximide have shown that IAA stimulated growth of rice coleoptile segments depend upon additional or new mRNA and protein synthesis.

To understand whether BR induced elongation growth of wheat coleoptile segments also depends on BR induced increase in RNA and protein synthesis, experiments were conducted using RNA and protein synthesis inhibitors.

Chloramphenicol, an inhibitor with aromatic ring and side chain similar to IAA is hypothesized to have similar chemical structure as that of 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) which is a competitive inhibitor of IAA (Muir, 1974). However, in coleoptile curvature bioassay chloramphenicol inhibits IAA induced response more than that of 2,4,5-T, indicating that it is a more potential inhibitor of auxin induced growth (Muir, 1974). This effect could be due to its competitive inhibition because of structural similarities with that of IAA, as well as due to its effect on protein synthesis. Chloramphenicol

effectively inhibited both IAA and BR induced coleoptie elongation in the present study.

Inhibitor of RNA biosynthesis, 5-fluorouracil was also effective in inhibiting both auxin and BR induced elongation. When coleoptile segments were incubated with IAA or BR along with 10 μM concentration of 5-FLU, 66 per cent inhibition of growth was observed (Fig. 24). Even pretreatment of coleoptile segments for 4 hours with 10 μM 5-FLU prior to the transfer to IAA or BR medium, was also effective in inhibiting the growth (Table 4a and 4b).

This data indicate that IAA as well as BR stimulated elongation of coleoptile segments depends on continuous RNA biosynthesis and preferentially the requirement for mRNA synthesis. Similarly, addition of different concentrations of cycloheximide from 0.1 μM to 1 mM progressively reduces IAA and BR stimulated elongation of coleoptile segments. At a concentration 10 μM of cycloheximide more than 50 per cent inhibition was observed (Fig. 25).Cycloheximide completely blocked IAA and BR stimulated growth at a concentration of 1mM.

Pretreatment for 4 hours with 10 μM cycloheximide also inhibited the IAA and BR stimulated elongation to the extent of 50 and 55 per cent, respectively.

These data suggest that enhanced growth of wheat coleoptile segments must be sustained by an increased

synthesis of mRNA and proteins, that may be associated with the deposition of new cell wall material. These results indicate that BR stimulated coleoptile growth also involves continuous synthesis of mRNA and proteins.

Few studies conducted to look in to the effect of BR on protein synthesis revealed that like auxins, BR induced growth responses also need continuous protein synthesis (Cerana et al., 1983b; Kalinich et al., 1985, 1986; Mandava et al., 1981). It is not clear at what levels BR treatment was shown to alter gene expression and synthesis of certain proteins (Braun and Wild, 1984). In soybean tissue, Clouse et al. (1990) reported that the in vitro synthesis of new polypeptides from RNA is induced by BR treatment. They also showed BR further promotes down-regulation of other proteins.

INFLUENCE OF ADENOSINE PHOSPHATE ON IAA AND BR INDUCED GROWTH

Cyclic AMP plays an important role in hormone action (Taiz and Zeiger, 1991). Adenosine phosphates, ADP and ATP also serve as sources of energy for energy dependent physiological and biochemical processes.

Providing energy rich compounds like ADP and ATP was expected to have some effect on action of IAA and BR, if the action is dependent upon metabolic processes like cell wall synthesis and protein synthesis. The results of

these experiments showed that AMP and ADP were not effective in enhancing IAA or BR induced growth further (Table 5a).

However, presence of ATP along with IAA or BR in the medium or pretreating the coleoptile segments with ATP resulted in stimulation of both IAA as well as BR induced growth indicating that energy dependent synthetic processes are involved in both IAA and BR induced cell elongation.

Potassium cyanide also inhibited both IAA as well as BR induced elongation growth in wheat coleoptile segments, indicating the necessity of energy in the form of ATP for growth. This information substantiate that both auxin as well as BR induced growth is an active growth and spend some supply of energy.

INFLUENCE OF BR ON ENDOGENOUS IAA CONCENTRATION AND AUXIN BINDING TO AUXIN RECEPTOR PROTEINS

Earlier studies by a number of workers (Clouse et al., 1992; Mandava, 1988 and Clouse and Zurek, 1991) indicated the possibility of BR, at least in part, changes the endogenous IAA concentrations resulting in stimulation of growth.

BR induced enhancement of auxin action can be explained in two ways. First, BR might be increasing the endogenous auxin concentration by increasing the

biosynthesis of auxin. Second, BR might be involved in sensitizing the tissue to auxin by increasing the affinity of auxin binding receptor protein to auxin. With an objective to probe into these two hypotheses, experiments were conducted using coleoptile tissue.

In the first set of experiments, polyclonal antibodies were developed for auxins by sensitizing rabbits with IAA-BSA conjugate. Standard procedures were followed to separate and identify the antibodies from rabbit serum. Qualitative and quantitative analysis like immunodiffusion and quantitative precipitation analysis showed the presence of specific antibodies against IAA-BSA conjugate. The membrane binding assay showed high specificity of the IAA-BSA IgG towards the hapten. In the indirect ELISA developed for quantification of IAA, antibody dilution of 1:40000 were found to be effective, suggesting the high specificity of antibody to the hapten. These antibodies were used to quantify endogenous auxin concentration in coleoptile tissue treated with auxin precursor and BR.

Coleoptile tip segments incubated in tryptophan medium showed little enhancement in endogenous IAA content. But, in the presence of BR, the endogenous auxin levels with and without tryptophan was high by 435 and 378 per cent compared to control at 4 hrs treatment. Similar increase was observed in response to GA in the medium

along with tryptophan. This data clearly indicated an increased IAA level in coleoptile tissue treated with BR, suggesting that at least in part, BR induced growth is due to enhanced IAA content in the coleoptile tissue. This could be attributed to increased conversion of tryptophan to IAA.

In another set of experiment, the influence of BR on binding of auxin to auxin binding proteins were determined using ^{14}C IAA. In the first experiment, the influence of BR on ^{14}C IAA uptake and content in coleoptile tissue were assessed by floating coleoptile segments in a medium containing IAA, ^{14}C IAA and different concentrations of BR. In the presence of BR, the ^{14}C activity in the coleoptile segments were higher indicating that IAA content in the tissue increases in the presence of BR.

In the second experiment, the membrane bound auxin binding proteins were isolated by adopting the procedure developed by Ray and Dohrman (1977). ^{14}C IAA was added to the medium, protein fraction and binding of ^{14}C IAA to auxin binding protein were studied in the presence of BR. ^{14}C IAA bound to the protein fraction was 16 per cent high compared to the treatment in which BR was excluded from the medium.

INVOLVEMENT OF CALCIUM AND CALCIUM CALMODULIN IN AUXIN AND BR INDUCED GROWTH

Calcium and calmodulin regulate biochemical processes and thus the growth and development of plants and animal systems is well documented by Cheung (1980) and Hanson (1983). In plants, hormonal as well as environmental stimuli induced growth and developmental responses were shown to be mediated by calcium and calmodulin (Leopold 1977; Poovaiah and Leopald 1973; Roux and Siocum, 1983). The involvement of calcium calmodulin protein in auxin induced elongation growth was shown by Elliott et al. (1983) and Raghothama et al. (1985). In the present investigation, the role of calcium and calmodulin in BR and IAA induced elongation was studied using various calcium inhibitors like CPZ, EGTA, lanthanum chloride, verapamil and ruthenium red.

Presence of calcium inhibitors in the medium along with IAA and BR inhibited IAA or BR induced elongation growth of the coleoptile tissue. Coleoptile tissue pretreated with calcium calmodulin inhibitor (CPZ), calcium channel blockers (RR, Verapamil, Lanthanum chloride) and calcium sequestering agent (EGTA) showed significantly reduced growth when test system is transferred to IAA or BR medium. This indicated that reducing endogenous free calcium concentration inside the cytosol reduces IAA as well as BR induced growth.

Coleoptile segments with high endogenous calcium concentrations showed high growth rate in the presence of auxin or BR in the medium. All the calcium inhibitors were also effective in inhibiting IAA or BR stimulated growth of coleoptile tissue with elevated calcium levels. These results emphasise two points. First, the IAA and BR induced growth was more in tissue having high cytosolic free calcium concentration. Second, calcium inhibitors were effective in reducing the hormone induced growth in tissue with elevated calcium concentration. The concentration range of calcium inhibitors which resulted in significant reduction in growth was comparable to the inhibition of other calcium or calmodulin mediated responses (Dieter and Marme 1981).

These results suggest the involvement of calcium and /or calcium calmodulin in auxin and BR mediated coleoptile elongation. IAA and BR may be increasing endogenous calcium concentration or actual content of calmodulin protein. It is also possible that in the presence of IAA or BR, the increased cytosolic calcium concentration may further enhance calcium or calmodulin mediated biochemical processes leading to higher elongation growth.

Experiments conducted to probe the involvement of calcium in IAA and BR induced growth brought out the following facts:

1) Coleoptile tissue with elevated cytosolic calcium concentration shows enhanced growth rates in the presence of IAA and BR.

2) Reducing the cytosolic free calcium content by blocking calcium channels or by sequestering free calcium or by inhibiting calmodulin protein, IAA or BR induced growth can be inhibited, suggesting the involvement of calcium in IAA and BR induced growth.

AGRICULTURAL APPLICATIONS

BRs have been shown to influence variety of growth and developmental processes in plant (Adam and Marquardt, 1985 and Meudt, 1987).

In as early as 1972, U.S. Department of Agriculture reported the effect of crude brassinolide extract of rape pollen on young bean plant and Siberian elm trees and found that brassins caused overall growth (Mitchell and Gregory, 1972).

Later, in a number of field studies the usefulness of brassinosteroids in increasing crop productivity has been shown in many vegetable and root crops like radish, lettuce, bean, pepper and potato (Maugh, 1981).

Yokota and Takahashi (1986) in their review article on agricultural application of brassinolide and related compounds in Japan, mentioned that seed treatment and

foliar application improved growth and productivity in a number of agricultural crops. Seed treatment was shown to improve productivity in rice and tobacco. Treatment of planting materials with brassinosteroid before planting was shown to improve yield in potato and sweet potato. Exogenous application of BRs to spikes and flowers were shown to improve yield in wheat, maize, rice and tomato. Yokota and Takahashi (1986) while referring to work conducted at Japan also indicated that the chemical was highly effective at low temperature. There are also claims that BRs enhances resistance to various stress such as cold, fungal infection, herbicide injury and salt (Hamada et al., 1985). It has also been shown that BR treated plants show resistance to chilling (Fujii et al., 1990; Katsumi, 1990).

In the present investigation, the influence of BR on cell elongation and leaf expansion was studied in horse gram and rice seedlings. Similarly, the influence of BR on fruit growth of grapes and yield in sunflower was investigated.

Experiments, conducted with horse gram seedlings showed that application of BR at concentration of 0.001 to 10 ppm to the cotyledonary leaves induced the development of a broad trifoliate leaf. The leaf area of the trifoliate leaf was more than three times that of untreated leaf. All the concentration of BR used in the

range of 0.001 to 10 ppm were effective in increasing the leaf area.

In rice seedlings also, exogenous application of BR (10 ppm) increased the area of the leaf which received BR, as well as the new leaf which was produced above the treated leaf. These experimental results indicated BR is effective in increasing cell expansion resulting in increased leaf expansion. This character of BR in increasing cell elongation and expansion found the way to promising areas to increase the agricultural productivity.

In three field experiments with grapes, the influence of BR and BR + GA on productivity was tested in two varieties of grapes Anab-E-Sahi and Dilkush. Immersing bunches during early developmental stage (15-20 days after flowering) in solution containing BR + GA resulted increased bunch weight in Anab-E-Sahi as well as in Dilkush. Treating bunches with GA alone was effective in increasing bunch weight. However, treating bunches with BR + GA increased the bunch weight further to an extent of 7 to 15 per cent over GA treatment alone. The individual berry weight and also berry volume showed marked increase. In one of the experiments with Anab-E-Sahi, the influence of BR, GA and BR + GA treatment on total soluble solids were estimated, the results indicated that the growth regulator treatment did not alter the TSS.

During fruit growth, the number of cells per fruit are decided at very early stage of development of the fruit. At later stage, the increase in weight and volume of the fruit is primarily due to increased cell expansion and accumulation of solutes in the cell. Hormones play a very important role in fruit set, fruit growth as well as in development of fruits. Auxins, gibberellins and cytokinins were shown to play a predominant role in cell division, cell expansion as well as in metabolization of solutes to developing fruits. Exogenous application of GA to increase bunch weight and berry size is a common practice in grapes and particularly in seedless varieties. Gibberellins were shown to increase cell elongation and expansion in fruit tissue. Our experimental results also correlated the results observed by other workers. GA was effective in increasing bunch weight and berry size. Treating bunches with BR + GA increased the bunch weight further. A number of basic studies conducted in the earlier part of the present investigation clearly proved that BR increases elongation of coleoptile tissue, hypocotyl and epicotyl segments by increasing cell elongation. In fruit tissue also BR might be eliciting similar response to increase the growth and weight of the berries.

In two other field experiments, the influence of foliar application of different concentrations of BR on growth and productivity of sunflower was

investigated. The first field experiment results indicated that foliar application of BR at 20th + 40th + 60th day increased the yield of sunflower by 15 per cent over control.

In the second field experiment conducted during summer season, a marginal increase in seed yield was observed in all the concentrations of BR treatment over control. However, the differences in yield between the treatments were not statistically significant.

These results on growth and productivity aspects in a few crop species indicate that brassinosteroids, in general, have a growth promoting effect. In grapes, brassinosteroid treatment increased the bunch weight, application of BR with GA promoted growth of bunches significantly more than that of GA alone in all the three experiments. Sasse(1990), while reviewing the work done on brassinosteroids indicated that BR promotes photosynthetic capacity and production at primary developmental stages, which led to accelerated growth of the whole plant. Meudt et al.(1983) conducted green house and field studies and noted acceleration and growth of barley and lettuce.

SUMMARY

VI SUMMARY

Brassinosteroids are plant growth promoting steroidal lactones. Many research groups have shown that BRs promote dual responses in plant tissues, namely, cell elongation and cell division leading to high growth rates and development. In the present investigations, experiments were conducted to study the role of BRs in plant growth processes. The main objectives of the study were to see the,

1. Influence of BRs on the growth responses in test systems;
2. Interaction of BR with auxins and other hormones on growth responses in a few test systems;
3. Mode of action of BR in induction of growth responses, and
4. Agricultural application of BRs in growth and productivity in grapes and sunflower.

INFLUENCE OF BRs ON GROWTH RESPONSES IN TEST SYSTEMS

The influence of BR on growth responses were studied in wheat coleoptile segment elongation, maize mesocotyl elongation, soybean stem elongation, mung bean seedling growth, mung bean epicotyl elongation, cucumber cotyledon expansion, coleus petiole curvature, tobacco leaf discs expansion, subabul pollen germination and pollen tube growth and dwarf rice leaf lamina inclination.

In wheat coleoptile segment, BR and IAA were effective in inducing elongation. The optimum concentration required to induce maximum response is very less for BR (0.2 μM) compared to IAA (10 μM). However, IAA induced elongation is more than that of BR. The optimum concentration required to induce maximum response is fifty times less in BR compared to IAA.

In soybean stem segments, the maximum elongation was obtained at 0.2 μM BR and 1 mM IAA concentrations. However, BR induced elongation was more and concentration required is five thousand times less than that of IAA.

In maize mesocotyl segments, the maximum elongation were obtained at 20 μM BR and 10 μM IAA concentrations. BR induced growth is less than IAA.

The influence of BR on mung bean seedling growth were studied. The treated hypocotyl and roots showed inhibitory effect of BR but in contrary, there was increase in radial expansion of hypocotyl.

The elongation response of mung bean epicotyl tissue to BR was studied and compared with that of GA. Similarly, the influence of BR and cytokinin (BA) in inducing expansion growth of cucumber cotyledons were compared. In mung bean epicotyl elongation test, the GA induced response was significantly high compared to BR. The

optimum concentration of BR required to induce maximum response was two times more than that of GA. This suggests that mung bean epicotyl elongation test is more sensitive to GA than BR. BR is not effective in cucumber cotyledon expansion test.

Application of BR to the cut ends of coleus petioles decreased the angle between the two petioles.

In expansion growth of tobacco leaf discs, a linear increase in fresh weight as well as diameter of discs were observed with increasing concentration of BR in the medium. BR also was effective in inducing pollen germination and pollen tube growth in subabul.

A micro-quantitative bioassay - dwarf rice leaf lamina inclination test, was standardized to quantify BRs. This bioassay was found highly specific to BR and effective in excised leaf segments and intact seedling systems. None of the other endogenous hormones were effective in eliciting responses in this test system. HBR also was equally effective in inducing inclination of rice leaf lamina.

INTERACTION OF BR WITH OTHER HORMONES IN FEW TEST SYSTEMS

In many test systems BR elicited responses similar to that of IAA. Based on this information a few experiments were conducted to see the interaction effect between BR and other hormones.

In wheat coleoptile segments, presence of both BR and IAA, in the medium resulted in more elongation growth than that of either BR or IAA alone. To know further about the interaction effect of IAA and BR, coleoptile segments were pretreated with BR for three hours and then transferred to IAA for post treatment. A synergistic effect of BR and IAA was observed at 0.2 μM BR and 10 μM IAA, respectively. The reverse sequence of treatment did not influence the BR induced elongation markedly. However, after pretreatment with BR for three hours, washing the coleoptile segments with water for two hours before transferring to IAA medium, significantly reduced the pretreatment effect of BR. This indicated that, pretreatment with BR increases IAA induced elongation growth.

In rice leaf lamina inclination test, IAA alone was not effective in leaf lamina bending. However, when IAA was applied with BR, the extent of leaf lamina bending was more than that of BR alone indicating an interaction effect of BR and IAA in this test system also.

In tobacco leaf disc expansion test, BR along with NAA acts synergistically in increasing fresh weight as well as expansion of leaf discs.

MODE OF ACTION OF BR

The time course of activity of BR in inducing elongation was compared with that of IAA at various

durations after incubation of coleoptile segments in the test solution. A linear increase in length of coleoptiles were observed in IAA treated tissues from ten minutes after incubation, till two hours, but not in BR. After two hours, growth was linear in both BR and IAA treated tissues. However, rate of increase was less in BR compared to IAA. The coleoptile segments floated in auxin solution, caused a measurable reduction in pH of the medium at two hours, with concomitant increase in growth. However, in BR medium, there was a late responses in growth as well as in reduction of pH of the media. When coleoptile segments were floated over media containing both IAA and BR, there was appreciable reduction in pH of the medium in a short period compared to IAA or BR alone in the medium and the growth was also more.

In order to find out essential differences in the mode of action of BR and IAA, experiments were done by using the inhibitors DCCD, KCN, galactose and protein synthesis inhibitors (5-fluorouracil, cycloheximide and chloramphenicol).

Experimental results using DCCD an inhibitor of membrane bound ATPase, inhibited both IAA and BR induced growth, which indicates that IAA and BR induced elongation response needs activity of ATPase proton pump on membranes for excretion of proton into cell wall, resulting in decrease in apoplastic pH.

Galactose was shown to inhibit auxin induced growth of avena coleoptile segments by inhibiting cell wall synthesis. In the present study galactose was effective in inhibiting both IAA and BR stimulated elongation of coleoptile segments. These results indicate that BR induced growth also involves synthesis of new cell wall materials.

Auxin induced cell elongation needs continuous mRNA and protein synthesis. To know whether BR stimulated cell elongation also needs mRNA and protein synthesis, the influence of RNA and protein synthesis inhibitors on BR induced growth was investigated. 5-Fluorouracil, is an RNA biosynthesis inhibitor, inhibited more than 60 per cent growth induced by IAA and BR. Cycloheximide and chloramphenicol - cytoplasmic protein synthesis inhibitors, also inhibited both IAA and BR stimulated growth of wheat coleoptiles. This explains that IAA and BR induced growth involves increase in RNA and protein synthesis.

The influence of metabolic energy source for IAA and BR induced wheat coleoptile growth has been investigated. The results indicated that IAA and BR showed a marked increase in cell elongation in the presence of ATP. The metabolic inhibitor - potassium cyanide was effective in reducing BR and IAA induced growth. It shows that BR and

IAA induced growth is an active and energy dependent process.

Many workers hypothesized that BR induced growth responses might take place through IAA by increasing IAA biosynthesis or by increasing the receptivity of IAA to Auxin binding proteins. BR induced IAA biosynthesis was tested using coleoptile tips, which contain all the enzymes necessary for IAA biosynthesis. Coleoptile tips incubated in tryptophan with or without BR and GA, showed a marked increase in growth. Diamedon - an IAA biosynthesis inhibitor, inhibits BR and GA induced growth in the presence of tryptophan. This suggested that both GA and BR increase elongation of coleoptile tip segments by increasing the conversion of tryptophan to IAA. To confirm this, IAA content was estimated in BR treated coleoptile tips at different intervals after incubation by using immunoassay technique (indirect ELISA).

For immunoassay technique, antibody was raised against IAA-BSA conjugate in rabbits. Immunodiffusion technique, quantitative precipitation analysis and membrane binding assay, showed that immunized rabbits have responded positively to hapten conjugate and produced specific antibodies for hapten and carrier protein BSA. The quantitative precipitation analysis and membrane binding assay showed 200 and 241 per cent of IgG, respectively, was specific to the hapten over

control. In indirect ELISA, crude serum is working at 1:40,000 dilution, it shows the high specificity of antibody to the hapten. These antibodies were used to quantify the endogenous IAA content in coleoptile tissue. Coleoptile tips treated with tryptophan show little increase in endogenous IAA content. In the presence of BR, the endogenous auxin level with or without tryptophan was high by 435 and 378 per cent compared to control at four hours treatment. Similar increase was observed in response to GA in the medium along with tryptophan. This infers that BR increases IAA content by increasing conversion of tryptophan to IAA.

In another set of experiment , the influence of BR on binding of auxin to ABPs were studied by using ^{14}C IAA. The coleoptile segments were incubated in ^{14}C IAA + cold IAA and ^{14}C IAA + cold IAA + BR. ^{14}C activity was more in IAA along with BR compared to IAA alone in all the fractions. This suggested that BR is increasing the binding of IAA to membrane fractions of the tissue. To confirm the above result, auxin binding proteins were isolated and incubated in ^{14}C IAA with BR. Presence of BR in the medium increased the binding of ^{14}C IAA to both IAA specific and nonspecific sites.

INVOLVEMENT OF CALCIUM AND CALCIUM CALMODULIN IN AUXIN AND BR INDUCED GROWTH

Involvement of calcium was observed in both IAA and BR induced elongation of coleoptile. In the presence of calcium inhibitors like, CPZ, EGTA, RR, $LaCl_3$ and verapamil in the medium along with IAA and BR, inhibited both IAA as well as BR induced growth of coleoptiles. Coleoptiles pretreated with calcium inhibitors showed less growth when transferred to BR or IAA in the medium. This indicated that reducing endogenous free calcium concentrations inside the cytosol reduces IAA as well as BR induced growth. All the calcium inhibitors were also effective in inhibiting IAA or BR stimulated growth of calcium enriched coleoptiles. The results suggest that involvement of calcium or calmodulin in auxin or BR mediated coleoptile elongation and BR mediated leaf lamina inclination in rice seedlings.

AGRICULTURAL APPLICATIONS

In the present study, the influence of BR on cell elongation and thus leaf expansion in horsegram and rice seedlings, and the influence of BR on fruit growth in grapes and yield of sunflower was investigated.

In horsegram seedlings, BR significantly increased leaf area and internodal elongation. BR also resulted in a marginal increase in leaf area in rice seedlings.

In field experiments with grapes, the influence of BR and BR along with GA on productivity was tested in two varieties of grapes, Anab-E-Sahi and Dilkush. Dipping the bunches in test solution containing BR along with GA (0.2 ppm BR + 25 ppm GA) was effective in increasing bunch weight and berry size more than that of GA or BR alone. BR in combination with GA increased bunch weight to the extent of 15 to 40 per cent over control and 7 to 15 per cent over GA treatment alone in Dilkush.

Foliar application of BR was not effective in increasing productivity in sunflower. Application of BR at a concentration of 1 ppm on 20th + 40th + 60th day after planting resulted in only a marginal increase in seed yield.

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[* = Originals not seen]