# THE EFFECT OF 6-BENZYLADENINE ON ADVENTITIOUS SHOOT FORMATION BY *LYCOPERSICON* SPECIES *IN VITRO*.

BY

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*We shall not cease from exploration*

*And the end of all our exploring*

*Will be to arrive where we started*

*And know the place for the first time.*

*Through the unknown, remembered gate*

*When the last of earth left to discover*

*Is that which was the beginning;*

*At the source of the longest river*

*The voice of the hidden waterfall*

*And the children in the apple-tree*

*Not known, because not looked for,*

*But heard, half-heard, in the stillness*

*Between two waves of the sea.*

T.S. Eliot, "Little Gidding"

# PREFACE

The experimental work described in this thesis was executed at the Vegetable and Ornamental Plant Institute, Pretoria and the Department of Botany, University of Natal, Pietermaritzburg from January 1992 to November 1993 under the supervision of Professor J. van Staden.

These studies except for the acknowledged work of others are the result of my own investigation.

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# ABSTRACT

*Lycopersicon esculentum* Mill. cv. Rodade was developed in South Africa for the fresh produce market. This cultivar is also of major importance for South African tomato breeding programmes because of its resistance to bacterial wilt. In this study, aspects of the effects of 6-benzyladenine on adventitious shoot formation by both *L. esculentum* cv. Rodade and *Lycopersicon peruvianum* Mill. were studied *in vitro*. These included the regeneration of adventitious shoots, the effects of different incubation times, the uptake and metabolism of BA and the effect of auxin on the metabolism of BA in both leaf and callus tissue of the two species.

Adventitious buds could be regenerated on all tissue types except for callus tissue of *L. esculentum*. A stepwise increase in the percentage shoots produced was observed indicating a period of induction wherein incubation on a medium containing BA is beneficial to the production of shoots. Leaf tissue was more responsive to BA treatments than callus tissue of both species. The main route of BA metabolism in both species is from BA to [9R]BA and [9R-MP]BA. Callus tissue of *L. esculentum* cv. Rodade however converted BA to the 3- and 9-glucosides of BA rather than to metabolically active forms of the cytokinin. The auxin, indole-3-acetic acid, played a definite role in the conversion of BA to [3G]BA and [9G]BA in leaf tissue of the tomato cultivar tested, but had no effect in callus tissue of this species.

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# CHAPTER 1

# INTRODUCTION

The tomato, *Lycopersicon esculentum* Mill., was cultivated by Indians in Mexico and introduced in Europe as early as the 16th century. Today the tomato is one of the major vegetable crops throughout the world. The tomato is often cited as an example of success in plant breeding and of potential for further improvement through the use of biotechnology.

The interest in tomato as a model system for genetic engineering is due in part to the large amount of work done on the genus *Lycopersicon* over the past 50 years. The work includes the collection of germplasm of *L. esculentum* and its wild relatives, the transfer of disease resistant genes from wild species and the development of a classical genetic map. Like other Solanaceae (*Petunia, Nicotiana*) the tomato is readily amenable to *in vitro* culture of various types of tissues and organs. As a result, new trends in basic tomato research have emerged which promise to be of enormous potential, also to practical breeding.

Frequently the efficiency of biotechnology through protoplast fusion, the transfer of genetic material to protoplasts and the transformation of single cells and callus are determined by the ability of tomato tissue cultures to regenerate shoots. Often these procedures result in the production of callus from which plants must be regenerated. Since most of these procedures are time consuming resulting in prolonged culture periods, shoots are seldom regenerated from young callus tissue. Although there have been some reports of the regeneration of shoots from long-term callus cultures it was also found that the ability to regenerate shoots from callus is lost in most tomato genotypes with prolonged culture periods.

The problem therefore lies with the regeneration of shoots from long term callus cultures of *L. esculentum.* If this problem could be solved, biotechnology methods could be implemented to assist in breeding programmes of almost any genotype of the cultivated tomato. This would make these methods important tools in plant breeding.

*Lycopersicon esculentum* cv Rodade is a South African bred cultivar which was released in 1982 for the fresh produce market. This cultivar plays a key role in current breeding programmes since it is resistant to bacterial wilt (*Pseudomonas solanacearum* (Smith) Smith), *verticillium* wilt (*Verticillium dahliae* Kleb.) and

strains 1 and 2 of *fusarium* wilt (*Fusarium oxysporum* f. *lycopersici* (Sacc.) S. & H.). This is of great importance to tomato producers in South Africa especially those whose fields are infested with bacterial wilt. It is therefore important to exploit the potential of this cultivar.

Upon comparison of *Lycopersicon esculentum* with a wild species such as *Lycopersicon peruvianum* Mill. it is noticable that the latter has a much higher regeneration potential. In *L. esculentum* regeneration can be achieved from leaf tissue, but not from callus in many cultivars. In *L. peruvianum* shoots are regenerated from leaf as well as callus tissue at a high frequency. This ability of *L. peruvianum* to regenerate readily has often been used in protoplast fusion experiments to improve the regeneration potential of *L. esculentum*. However, this is not always the most effective method of improving regeneration potential since all characteristics are transferred and good as well as bad traits are expressed in fusion products. It is therefore important to investigate the factors that control regeneration in order to obtain a solution for the problem of regenerating plantlets from tomato callus cultures.

The aim of this research was to investigate differences in the metabolism of 6-benzyladenine (BA) in leaf and callus tissue of *L. esculentum* cv. Rodade and *L.*

*peruvianum* in order to understand problems related to regeneration from callus cultures of *L. esculentum.* Throughout the experiments both leaf and callus tissue of *L. esculentum* and *L. peruvianum* were tested in order to make a comparison between these tissues.

Initially the conditions for the regeneration of adventitious shoots from leaf and callus tissue from both species were determined in order to establish the correct media compositions to be used in the subsequent experiments. In order to determine the effects of different incubation periods, the effects of culture in the presence of BA over 40 days on solid and in liquid medium on the induction of adventitous shoots on leaf and callus tissue of both *L. esculentum* and *L. peruvianum* was determined. The effects of cultivation on media with different high BA concentrations at pulse intervals from 0 to 6 hours as well as from 1 to 40 days were also investigated in leaf and callus tissue of both species.

The uptake and metabolism of BA in leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were investigated in order to determine the effectivity of BA in a tissue culture system such as the one under investigation. Finally the effect of an auxin (IAA) on the metabolism of BA was investigated in all four tissue types to determine the effect of this auxin on BA metabolism during organogenesis.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 INTRODUCTION

The tomato was cultivated by Indians in Mexico and introduced into Europe as early as the 16th century. It was not until the second half of the nineteenth century that the tomato, *Lycopersicon esculentum* Mill., became recognised as a highly valuable nutritious food crop. Today the tomato is one of the major vegetable crops throughout the world with a production of approximately 50 million metric tons in 1986 (HILLE, KOORNNEEF, RAMANNA and ZABEL, 1989).

The commercial tomato is a member of a relatively small genus, *Lycopersicon*, within the family Solanaceae. The genus consists of nine species which can be divided into two species complexes. The esculentum complex consists of *L. esculentum* Mill., *L. cheesmanii* Riley, *L. hirsutum* HBK, *L. pennellii* W.G. D'Arcy, *L. chmielewskii* Rick *et al.* and *L. parviflorum* Rick *et al.* and the peruvianum

complex which consists of *L. peruvianum* Mill. and *L. chilense* Dun. Both complexes served as an invaluable source of genetic variation and disease resistant genes used for improving the cultivated tomato (STEVENS and RICK, 1986).

A wealth of genetic information and knowledge has been gathered on the tomato. With the development of DNA recombinant technology, restriction fragment length polymorphism mapping (RFLP) (HELENTJARIS, SLOCUM, WRIGHT, SHAEFER and NIENHUIS, 1986; ZAMIR and TANKSLEY, 1988), effective DNA transformation as well as cell and tissue culture techniques, the tomato has become amenable to cellular and molecular analysis and manipulation. As a result, new trends in basic tomato research emerged which promise to be of enormous potential, also to conventional breeding.

As other members of the Solanaceae, such as *Nicotiana* and *Petunia*, the tomato is relatively amenable to *in vitro* culture. Various types of tissues and organs can be employed in this type of experimentation. Procedures have been developed for the *in vitro* culture of embryos, shoots, roots, leaf explants and protoplasts. These methods have opened the possibilities for various biotechnological applications, e.g. embryo rescue, *in vitro* mutant selection, basic biochemical and virus research, the isolation of mitotic chromosomes, protoplast fusion and genetic transformation.

The tomato cultivar Rodade became commercially available in 1984 and is one of the latest developed cultivars in South Africa (BOSCH and OLIVIER, 1985). The cultivar is resistant to *Pseudomonas solanacearum* (Smith) Smith, races 1 and 2 of *Fusarium oxysporum* f. *lycopersici* (Sacc.) S. & H. and also to *Verticillium dahliae* Kleb. The plant has a determinate growth habit, is jointless (which facilitate the picking process) and the fruit are less prone to abscission. These traits make cultivar Rodade very important in the Republic of South Africa. Due to its resistance to bacterial wilt it is also of international importance.

## 2.2 CALLUS AND SUSPENSION CULTURES

The most important factor in successfully establishing and maintaining callus cultures has been the genotype. This was recognised in early studies by ULRICH and MACKINNEY (1969) where genotypes responded differently to levels of nutrients and growth regulators or inhibitors in the medium. DE LANGHE and DE BRUIJNE (1976) observed that *L. peruvianum* explants were much more vigorous in their growth compared to *L. esculentum* varieties. *L. peruvianum* responded to variable auxin and cytokinin ratios while *L. esculentum* did not. This response in *L. esculentum* was thought to be caused by an imbalance of gibberellic acid ($GA_3$) in the original explant. Treatment of plants with a GA-synthesis inhibitor

2-chloroethyl-trimethyl-ammonium chloride (CCC), increased callus and shoot formation of *L. esculentum* tissue explants. Callus of *L. esculentum, L. peruvianum* and *Solanum pennellii* responded differently to similar media (TAL, HEIKIN and DEHAN, 1978). *S. pennellii* produced six times more callus than *L. esculentum* and twice more than *L. peruvianum*. KOBLITZ and KOBLITZ (1982a) noted different media requirements for callus formation from various *Lycopersicon* species. *L. esculentum* cv. Lukullus and *L. pimpinellifolium* grew best on B5 medium (GAMBORG, MILLER and OJIMA, 1968). *L. peruvianum* and several other *L. esculentum* cultivars grew best on a MURASHIGE and SKOOG (1962) (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN).

A review of the media used for the initiation and maintenance of callus (Table 2.1) showed that the basal salt formulation used with most success was the MS nutrient medium. Various organic nutrients such as amino acids, vitamins and inositol were used but no studies showed conclusively that they were necessary. Media such as R3B (KOORNNEEF, VAN DIEPEN, HANHART, KIEBOOM-DE WAART, MARTINELLI, SCHOENMAKERS and WIJBRANDI, 1989), the modified medium of White (CHIN, HAAS and STILL, 1981) and DBM1 (JARAMILLO and SUMMERS, 1990) were also used for the production of callus. The growth regulators were the most important requirements for callus formation. The general

rule of high auxin/cytokinin ratio held for callus production.

The auxins used most frequently were 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and 2,4-D at levels from 0.1 to 5.0 mg $dm^{-3}$. The cytokinins preferred were either kinetin or BA at levels of 0.1 to 5.0 mg $dm^{-3}$ (Table 2.1). It is also evident that callus could be maintained either in light or in the dark at 25°C. KATZ and TAL (1980) found that callus could be induced from tissue of *L. esculentum* and *L. peruvianum* under identical hormonal conditions on identical media. Evidence also suggested that almost any tissue type could be used to induce callus (Table 2.1). Tissue such as leaf and stem was very popular. Cotyledon, hypocotyl, root and anther tissue were also used frequently. GARCIA-REINA and LUQUE (1988) found that tomato roots produced white friable callus, whereas shoot tissue produced nodular green callus. Tomato leaf tissue also produced white friable callus (BEHKI and LESLEY, 1980) which shows that the existence of chlorophyll in the explant tissue does not determine the production of cholorphyll in callus tissue.

**TABLE 2.1** : Data on callus culture of tomato.

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | hypocotyl | Pea extr. | - | dark, 25°C | ULRICH and MACKINNEY, 1969 |
| *L. esculentum* | stem | MS | NAA (1.0)<br>BA (1.0) | dark, 26°C | WARREN and ROUTLEY, 1970 |
| *L. esculentum* | leaf | MS | IAA (2.0 - 4.0)<br>KIN (2.0 - 4.0) | dark, 25°C | PADMANABHAN, PADDOCK and SHARP, 1974 |
| *L. esculentum* | leaf | MS | NAA (2.0)<br>IAA (0.5)<br>KIN (0.1) | dark, 26°C | LEVENKO and KIFORAK, 1975 |
| *L. esculentum* | hypocotyl | MS | NAA (5.0)<br>BA (1.0) | light, 25μmol $m^{-2}$ $s^{-1}$, 25°C | IMANISHI and HIURA, 1976 |

* Various concentrations used

**TABLE 2.1** : Data on callus culture of tomato (Continued).

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | hypocotyl | B5 | - | shaker 110 rpm | ELLIS, 1978 |
| *L. esculentum* | leaf | MS | NAA (0.5)<br>KIN (0.5) | light,16h, 25°C | TAL, HEIKIN and DEHAN, 1978 |
| *L. esculentum* | stem | MS | NAA (2.0)<br>IAA (2.0)<br>KIN (1.0) | - | DANEK-JEZIK and SCHMIDT, 1979 |
| *L. esculentum* | stem, leaf anther | MS | NAA (2.0)<br>BA (1.0) | dark, 25°C | MEREDITH, 1979 |
| *L. esculentum* | leaf | MS | NAA (0.5)<br>KIN (0.5) | light 16h/dark 8h, 25°C | KATZ and TAL, 1980 |

* Various concentrations used

**TABLE 2.1 :** Data on callus culture of tomato (Continued).

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | hypocotyl | MS | 2,4-D* | dark, 25°C | LIEBISCH, 1980 |
| *L. esculentum* | stem | White | BA (0.22)<br>NAA (11.0) | dark, 27°C | CHIN,HAAS and STILL, 1981 |
| *L. esculentum* | leaf | MS | BA (0.5)<br>IAA (0.5)<br>2,4-D (1.0) | dark, 25°C | ZAPATA, SINK and COCKING, 1981 |
| *L. esculentum* | hypocotyl | MS | 2,4-D (1.0)<br>KIN (0.02) | dark, 25°C | NOVER, KRANZ and SCHARF, 1982 |
| *L. esculentum* | hypocotyl | MS | 2,4-D (0.5)<br>KIN (0.1) | dark, 25°C | RANADE and DAVID, 1985 |

* Various concentrations used

**TABLE 2.1 :** Data on callus culture of tomato (Continued).

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | leaf | R3B** | NAA (2.0)<br>BA (1.0) | light 16h/dark 8h,<br>25°C | KOORNNEEF, HANHART and MARTINELLI, 1987 |
| *L. esculentum* | cotyledon | MS | BA*<br>IAA* | light 16h/dark 8h,<br>25 µmol $m^{-2}$ $s^{-1}$, 25°C. | GARCIA-REINA and LUQUE, 1988 |
| *L. esculentum* | leaf | R3B** | NAA (2.0)<br>BA (1.0) | light 16h/dark 8h,<br>25°C | KOORNNEEF, VAN DIEPEN, HANHART, KIEBOOM-DE WAART, MARTINELLI, SCHOENMAKERS and WIJBRANDI, 1989 |
| *L. esculentum* | leaf | MS | 2,4-D (0.5)<br>BA (1.0) | light,50 µmol $m^{-2}$ $s^{-1}$ | TOYODA, SHIMIZU, CHATANI, KITA, MATSUDA and OUCHI, 1989 |

* Various concentrations used
** Code used by authors for modified MS medium (R3B)

**TABLE 2.1 :** Data on callus culture of tomato (Continued).

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | anther | DBM1*** | NAA (2.0)<br>KIN (5.0) | dark, 25°C | JARAMILLO and SUMMERS, 1990 |
| *L. esculentum* | cotyledon | MS | IAA (2.0 - 4.0)<br>KIN (2.0 - 4.0) | dark, 25°C | COMPTON and VEILLEUX, 1991 |
| *L. esculentum* | anther | MS | NAA (2.0)<br>KIN (5.0) | dark, 25°C | JARAMILLO and SUMMERS, 1991 |
| *L. peruvianum* | roots | White | KIN (1.0)<br>2,4-D (1.0)<br>BA (1.0) | dark, 25°C | NORTON and BOLL, 1954 |

* Various concentrations used
*** Code used by authors for modified MS medium (DBM1)

**TABLE 2.1** : Data on callus culture of tomato (Continued).

| Species cultured | Explant used | Salts | Hormones ($mg.dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. peruvianum* | stem | MS | NAA (0.5)<br>KIN (0.5) | light,16h, 25°C | TAL, DEHAN and HEIKIN, 1977 |
| *L. peruvianum* | stem | MS | NAA (0.5)<br>KIN (0.5) | light 16h/dark 8h | KATZ and TAL, 1980 |
| *L. peruvianum* | cotyledon | MS | IAA (0.17)<br>BA (2.25) | dark, 25°C | KOBLITZ and KOBLITZ, 1982b |
| *L. peruvianum* | cotyledons | MS | 2,4-D (1.0)<br>KIN (0.02) | dark, 25°C | NOVER, KRANZ and SCHARF, 1982 |
| *L. pimpinelli-folium* | stem | MS | NAA (2.0)<br>BA (1.0) | dark, 25°C | MEREDITH, 1979 |

* Various concentrations used

Suspension cultures could be established readily from tomato callus. The best suspension cultures were established from callus cultures that were fast-growing and translucent in appearance (NOVER, KRANZ and SCHARF, 1982). In some cases it took up to 40 subcultures of the callus to obtain the pale grey colour typical of callus suitable for the initiation of a cell suspension culture (KOBLITZ and KOBLITZ, 1982a). Suspension cultures often also had a pale grey colour (ELLIS, 1978). These suspension cultures consisted of single cells, short filaments and aggregates (BREIDENBACH and WARING, 1977). Cultures are normally transferred at 2 to 14 day intervals (NOVER, KRANZ and SCHARF, 1982). Media requirements for suspension cultures are similar to those for callus culture with no other special formulations reported.

## 2.3 SHOOT REGENERATION

Regeneration of adventitious shoots in leaf tissue was achieved as early as 1954 by NORTON and BOLL. An important addition to their medium was 40 mg $dm^{-3}$ adenine (Ade). KARTHA, CHAMPOUX and PAHL (1977) showed that many combinations of auxins and cytokinins would allow shoot development in tissue cultures of *L. esculentum* cv. Starfire. There was no suggestion of multiple shoot formation from shoot tip explants. Three lines of *L. esculentum* showed

regeneration of adventitious shoots from leaf derived callus (PADMANABHAN, PADDOCK and SHARP, 1974). This regeneration was achieved on an MS medium supplemented with 4.0 mg $dm^{-3}$ IAA. Multiple adventitious shoots were produced in tissue cultures of *L. esculentum* cv. Moneymaker (NOVAK and MASKOVA, 1979).

Upon closer examination of specific growth regulators used it was found that combinations of IAA and BA were superior to IAA and kinetin (GUNAY and RAO, 1980) for shoot regeneration. Benzyladenine or zeatin (Z) alone could induce shoots (KARTHA, GAMBORG, SHYLUK and CONSTABEL, 1976) and zeatin and BA were superior to kinetin for shoot regeneration from leaf explants (DHRUVA, RAMAKRISHIVAN and VAIDYANATHAM, 1978). These studies also showed that the use of NAA to initiate callus could be detrimental to shoot formation. Increased shoot formation was noted when the anti-auxin 2,3,4-triiodobenzoic acid (TIBA) was used (CASSELS, 1979). UDDIN, BERRY and BISGES (1988) found kinetin to be equal to zeatin in terms of shoot induction in 10 processing tomato cultivars. Shoot formation could also be increased by the addition of CCC to the plant (DE LANGHE and DE BRUIJNE, 1976). BEHKI and LESLEY (1980) found an induction as well as a differentiation period and a critical ratio of ammonia to nitrate essential to the regeneration of tomato shoots *in*

*vitro*. Zeatin as a single requirement for shoot regeneration was demonstrated by MONTAGNO, LINEBERGER and BERRY (1989).

Long-term callus cultures of *L. esculentum* were claimed to regenerate shoots on a medium with 1.0 mg $dm^{-3}$ NAA (IMANISHI and HIURA, 1976). HERMAN and HAAS (1978) found that long-term callus will only regenerate shoots if the original explant containing evidence of organization remained attached to the callus. Once removed, the callus would not regenerate. SCHUTZE, LEIKE, MALTZ and TAIRBEKOV (1987) subjected cell suspension cultures of the cultivar Nadja to weightlessness in the Soviet biosatelite KOSMOS 1667. No differences were found in cultures subjected to these conditions when compared to control cultures on earth with regard to regeneration frequency. However, a higher number of shoots were found after space flight.

A comparison of morphogenetic response between *L. esculentum* and *L. peruvianum* (DE LANGHE and DE BRUIJNE, 1976) showed that shoot formation in *L. esculentum* was 2 to 3 times lower that in the wild species. TAL, DEHAN and HEIKIN (1977) also found that *L. peruvianum* had a higher regeneration potential than *L. esculentum*. Attempts to transfer the regeneration potential of *L. peruvianum* to *L. esculentum* were successful (THOMAS and PRATT, 1982a)

demonstrating the possibility to increase the regeneration potential of *L. esculentum* by transferring desirable traits from wild species to the cultivated tomato. Despite these results it is clear that *L. esculentum* also differs greatly between cultivars and other *Lycopersicon* species in its requirements for regeneration (Table 2.2). BEHKI and LESLEY (1976) showed that of 15 different cultivars of tomato tested for morphogenetic potential, 12 differed in their requirements for regeneration. The difficulty of finding the correct media for optimum culture was also demonstrated by the work of VNUCHKOVA (1977) who tested 150 different variants of media for its ability to induce callus and shoots *in vitro*. GARCIA-REINA and LUQUE (1988) showed a difference between the morphogenetic potential of different tomato cultivars. A difference in morphogenetic potential between different tissue types of tomato was also demonstrated by the work of COMPTON and VEILLEUX (1991). Direct regeneration of adventitious shoots on explants incubated on a medium with high cytokinin/auxin ratios have been reported by many authors. In general the observations of KARTHA, GAMBORG, SHYLUK and CONSTABEL (1976) that media with BA or zeatin at concentrations between 1.0 to 5.0 mg $dm^{-3}$ combined with IAA concentrations between 0.1 to 0.5 mg $dm^{-3}$ were most effective for shoot regeneration, has been confirmed by various authors (Table 2.2). In *L. esculentum* shoot formation is often accompanied by the production of callus whereas in *L. peruvianum* shoots often appear directly on differentiated tissue. It

is obvious that a range of media are adequate for the regeneration of shoots from tomato tissue and that the regeneration potential is controlled by the genetic background as well as the age and the physiological state of the tissue (Table 2.2).

**TABLE 2.2** : Data on the regeneration of adventitious shoots in *Lycopersicon* species.

| Species cultured | Tissue type used | Media | Hormones (mg $dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | leaf callus | MS | IAA•, KIN• | light 12h/dark12h, 22°C | PADMANABHAN, PADDOCK and SHARP, 1974 |
| *L. esculentum* | leaf | MS | NAA• BA• | light 16h/dark 8h, 20 µmol $m^{-2}$ $s^{-1}$, 22°C | BEHKI and LESLEY, 1976 |
| *L. esculentum* | stem callus | LS | IBA•, IAA• KIN• | light, 25 µmol $m^{-2}$ $s^{-1}$, 25°C | DE LANGHE and DE BRUIJNE, 1976 |
| *L. esculentum* | leaf | MS | IAA•, NAA• KIN• | light 16h/dark 8h, 30 µmol $m^{-2}$ $s^{-1}$, 27°C | TAL, DEHAN, HEIKIN, 1977 |
| *L. esculentum* | cotyledon leaf | MS | IAA•, KIN• Ade• | 24-26°C | VNUCHKOVA, 1977 |

• Various concentrations used.

**TABLE 2.2** : Data on the regeneration of adventitious shoots in *Lycopersicon* species (Continued).

| Species cultured | Tissue type used | Media | Hormones (mg $dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | leaf | MS | IAA (4.0)<br>KIN (4.0) | light 12h/dark 12h,<br>20 μmol $m^{-2}$ $s^{-1}$ | HERMAN and HAAS, 1978 |
| *L. esculentum* | hypocotyl<br>cotyledon | MS | IAA•, BA•<br>IAA•, KIN• | light,20 μmol $m^{-2}$ $s^{-1}$,<br>25°C | GUNAY and RAO, 1980 |
| *L. esculentum* | leaf | MS | Z (2.0) | light 16h/dark 8h,<br>25 μmol $m^{-2}$ $s^{-1}$,<br>25°C | KOORNNEEF, HANHART and MARTINELLI, 1987 |
| *L. esculentum* | cell cultures | MS | Z (2.25)<br>BA (0.45)<br>IAA (0.17) | light 16h/dark 8h,<br>14 μmol $m^{-2}$ $s^{-1}$,<br>25°C | SCHUTZE, LEIKE, MALTZ and TAIRBEKOV, 1987 |

• Various concentrations used.

**TABLE 2.2** : Data on the regeneration of adventitious shoots in *Lycopersicon* species (Continued).

| Species cultured | Tissue type used | Media | Hormones (mg $dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | callus | MS | BA*, IAA* 2,4-D* | light,25 μmol $m^{-2}$ $s^{-1}$ | GARCIA-REINA and LUQUE, 1988 |
| *L. esculentum* | crown gall | R3B | None | light 16h/dark 8h, 10 μmol $m^{-2}$ $s^{-1}$, 25 - 30°C | NECASEC, DUSBABKOVA and PEKARKOVA-TRONICKOVA, 1988 |
| *L. esculentum* | cotyledon | MS | IAA (1.0) KIN (1.0) | light 12h/dark 12h, 25°C | UDDIN, BERRY and BISGES, 1988 |
| *L. esculentum* | leaf | MS | Z (2.0) | light 16h/dark 8h, 25 μmol $m^{-2}$ $s^{-1}$ | KOORNNEEF, VAN DIEPEN, HANHART, KIEBOOM-DE WAART, MARTINELLI, SCHOENMAKERS and WIJBRANDI, 1989 |

* Various concentrations used.

**TABLE 2.2** : Data on the regeneration of adventitious shoots in *Lycopersicon* species (Continued).

| Species cultured | Tissue type used | Media | Hormones (mg $dm^{-3}$) | Culture conditions | Reference |
|---|---|---|---|---|---|
| *L. esculentum* | cotyledons | MS | Z (2.0) | light 16h/dark 8h, 33 µmol $m^{-2}$ $s^{-1}$, 25°C | MONTAGNO, LINEBERGER and BERRY, 1989 |
| *L. esculentum* | epicotyl | MS | BA (1.13) | light 16h/dark 8h, 50 µmol $m^{-2}$ $s^{-1}$ 25°C | COMPTON and VEILLEUX, 1991 |
| *L. peruvianum* | stem callus | LS*** | IBA*,**, IAA* KIN* | light, 25 µmol $m^{-2}$ $s^{-1}$, 25°C | DE LANGHE and DE BRUIJNE, 1976 |
| *L. peruvianum* | leaf | MS | IAA*, NAA* KIN* | light 16h/dark 8h, 30 µmol $m^{-2}$ $s^{-1}$, 27°C | TAL, DEHAN and HEIKIN, 1977 |

* Various concentrations used.
** 3-indolebuteric acid
*** LINSMAIER and SKOOG nutrient medium (LS) (LINSMAIER and SKOOG, 1965)

## 2.4 THE INFLUENCE OF OTHER FACTORS ON ORGANOGENESIS

The organogenetic process in various plant species have been investigated using tissue transfer experiments (CHRISTIANSON and WARNICK, 1983). The influence of genotype, explant type, subculture interval and environmental conditions (KABURU M'RIBU and VEILLEUX, 1990) as well as explant size and configuration (BECK and CAMPER, 1991) have recently been studied with respect to organogenesis. It was found that leaf discs could be removed from BA-containing media and still produce shoots on media without BA, that shoot formation was influenced by subculture interval and that regular subculture of stock plantlets was essential for good regeneration. It was also shown by ATTFIELD and EVANS (1991) that explant tissue became determined for shoot production after a period of exposure to a shoot inducing medium. Liquid media and high BA pulses were also investigated (CHRAIBI, CASTELLE, LATCHE, ROUSTAN and FALLOT, 1992; GOLDFARB, HOWE, BAILEY, STRAUSS and ZAERR, 1991). Liquid media enhanced shoot regeneration in some tissue types and solidifying agents played an important role in the availability of nutrients to tissue *in vitro* (JARAMILLO and SUMMERS, 1990).

## 2.5 ISOLATION AND CULTURE OF PROTOPLASTS

The first enzymatic isolation of plant protoplasts was from root tip cells of *L.esculentum* cv. Best of All (COCKING, 1960). Protoplasts were also isolated from tomato fruit placental tissue (COCKING and GREGORY, 1963). GREGORY and COCKING (1965) subsequently found that a large number of protoplasts could be isolated by using a commercial pectinase enzyme. Subsequent studies were focused on the isolation of leaf protoplasts and their culture. CASSELS and GATENBY (1975) first reported the release of tomato leaf protoplasts using a one-step enzymatic method. CASSELLS and BARLASS (1976) conducted a physiological study of winter vs. summer grown greenhouse tomatoes. It was found that leaves of plants grown under summer conditions had a tenfold higher level of calcium pectate than those of winter-grown plants. A reduced protoplast release was therefore found in summer-grown plants. The effects of several pre-treatments of donor plants relative to viable protoplast release were studied by TAL and WATTS (1979). It was found that plants grown in 82% relative humidity (RH) showed a higher release of protoplasts than did plants grown at 56% RH. NIEDZ and SINK (1988) did a multifactor analysis of the effects of preconditioning on protoplast viability. They showed that an optimum response was achieved with a pre-treatment of 48h darkness and 12h cold treatment at 4°C.

Leaves of 4 to 6-week-old tomato plants have frequently been used as donor material for isolating protoplasts and for obtaining cell division in culture. Protoplasts have been successfully isolated and cultured from cotyledons, leaf tissue and cell suspensions (Table 2.3).

With respect to enzymes, most researchers use a combination of cellulase, pectinase and driselase for the release of tomato protoplasts (Table 2.3).

Although media such as those of White (PONJAR and COCKING, 1968), (B5) (GAMBORG, MILLER and OJIMA, 1968) and coconut water (POJNAR and COCKING, 1968) were used, a definite trend towards using MS or modified MS-media are found up to 1981 (Table 2.3). The use of different media remained cultivar specific and it was not until 1985 that SHAHIN developed a range of media suitable for the isolation and culture of tomato protoplasts (Table 2.3). SHAHIN (1985) showed that his formulations could support the isolation and culture of protoplasts of 14 tomato cultivars.

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species.

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treatment | Reference |
|---|---|---|---|---|---|---|
| *L. esculentum* | locule | Pectinol-R10 | White or Coconut water | cell aggregate | - | POJNAR and COCKING, 1968 |
| *L. esculentum* | leaf | 4.0% Meicelase-P<br>0.4% Macerozyme<br>0.25% Driselase | B5 + 24D (1.0)+<br>NAA (0.5) + BA(0.5) | callus<br>plants | -<br>- | ZAPATA, EVANS, POWER, and COCKING, 1977 |
| *L. esculentum* | cotyledon | 0.5% Onozuka S5<br>0.5% Macerozyme<br>0.1% Driselase | Modified Kao (KAO, 1977) + BA (0.5) + KIN* | plants | 20h; Dark; 25°C | KOBLITZ and KOBLITZ, 1982a |
| *L. esculentum* | cell suspension | 0.25% HuPc-cellulase | Modified MS + IAA (1.0) + NAA (1.25) + 24D (0.5) + KIN (0.5) + BA (0.25) | shoots | 20h; Dark; 25 °C | KOBLITZ and KOBLITZ, 1982b |

* Various concentrations used.

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species (Continued).

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treatment | Reference |
|---|---|---|---|---|---|---|
| *L. esculentum* | cell suspension | 1.0% Driselase<br>0.1% HuPc-cellulase | Modified MS + 24-D (1.0) + KIN (0.02) | cell division | - | TEWES, GLUND, WALTHER and REINBOTHE, 1984 |
| *L. esculentum* | leaf | 0.1% Meicelase<br>0.1% Macerase | Modified MS + 2,4D (1.0) + NAA (1.0) | plants | - | NIEDZ, RUTTER, HANDLEY and SINK, 1985 |
| *L. esculentum* | leaf | 0.2% Pectinol Ac<br>1.5% Cellulysin | JSR** see Reference | shoots | - | O'CONNELL and HANSON, 1985 |
| *L. esculentum* | leaf | 0.1% Macerozyme<br>0.75% Cellulysin | TM-4 | plants | 2 days; Dark; 25 °C | SHAHIN, 1985 |

* Various concentrations used.
** Code used by authors for modified MS medium (JSR).

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species (Continued).

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treatment | Reference |
|---|---|---|---|---|---|---|
| *L. esculentum* x *L. peruvianum* | leaf | 0.6% Cellulase<br>0.2% Macerozyme | TM-2<br>TM-3<br>CPW | shoots | 0.5 MS<br>Dark, 4°C | KOORNNEEF, HANHART and MARTINELLI, 1987 |
| *L. esculentum* | leaf | 0.6% Cellulysin<br>0.1% Macerase | Modified B5 + NAA (1.0) + BA (0.5) + 24D (0.5) | plants | 18h;Dark;20°C<br>6h; Dark; 4°C | TAN, RIETVELD,VAN MARREWIJK and KOOL, 1987 |
| *L. esculentum* | leaf | 3.0% Meicelase<br>0.1% Macerase | 8E | shoots | - | NIEDZ and SINK, 1988 |
| *L. esculentum* x *S. lycopersicoides* | stem | 1.2% Cellulase<br>0.6% Macerase | SCM** | plants | Dark; 4 °C | GLEDDIE, KELLER and POYSA, 1989 |
| *L. esculentum* | leaf | 0.5% Cellulase<br>0.25% Macerozyme | TM-4<br>(SHAHIN, 1985) | plants | 1h;Dark;20°C | DERKS, ZELCER and COLIJN-HOOYMANS,1990 |

* Various concentrations used.

** (TAN, RIETVELD, VAN MARREWIJK and KOOL, 1987)

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species (Continued).

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treat-ment | Reference |
|---|---|---|---|---|---|---|
| *L. esculentum* | leaf | 0.2% Macerozyme<br>1.0% Cellulase<br>0.5% Driselase | TM-2 | callus | 18h at 4°C<br>6h at 25°C | BELLINI, CHUPEAU, GERVAIS,VASTRA and CHUPEAU, 1990 |
| *L. esculentum* | leaf | 0.1% Cellulase<br>0.015% Macerozyme | TM-2<br>TM-3 | plants<br>plants | 0.5 MS-salts<br>Dark, 4°C | WIJBRANDI, CAPELLE, HANHART,VAN LOENEN MARTINET-SCHURINGA and KOORNNEEF, 1990 |
| *L. esculentum* | leaf | 0.4% Cellulase<br>0.2% Driselase | TM-3<br>TM-4 | plants<br>plants | -<br>- | RATUSHNYAK, LATYPOV, SAMOYLOV, PIVEN and GLEBA, 1991 |
| *L. esculentum* | fruit | 0.02% Pectolyase<br>0.5% Macerozyme<br>1.0% Cellulase | - | - | - | FIEUW and WILLENBRINK, 1991 |

* Various concentrations used.

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species (Continued).

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treatment | Reference |
|---|---|---|---|---|---|---|
| *L. peruvianum* | leaf | 4.0% Meicelase-P<br>0.4% Macerozyme<br>0.25% Driselase | B5 + 24D (1.0)+<br>NAA (0.5) + BA(0.5) | callus<br>plants | -<br>- | ZAPATA, EVANS, POWER, and COCKING, 1977 |
| *L. peruvianum* | leaf | 0.5% macerozyme | MS | shoots | 1h in mannitol | MUHLBACH, 1980 |
| *L. peruvianum* | leaf | 1.0% Cellulose-R10<br>0.5% Macerase<br>0.5% Driselase | MS + Kinetin (1.0)<br>+ NAA (1.0) | plants | - | ZAPATA and SINK, 1981 |
| *L. peruvianum* | leaf | 0.1% Cellulase<br>0.015% Macerozyme | TM-2<br>TM-3 | plants<br>plants | 0.5 MS-salts<br>Dark, 4°C | WIJBRANDI, CAPELLE, HANHART, VAN LOENEN MARTINET-SCHURINGA and KOORNNEEF, 1990 |

* Various concentrations used.

**TABLE 2.3 :** Protoplast isolation, culture and regeneration of *Lycopersicon* species (Continued).

| Species | Cell source | Enzyme mixture | Culture Medium ($mg.dm^{-3}$) | Growth response | Pre-treatment | Reference |
|---|---|---|---|---|---|---|
| *L. peruvianum* | leaf | 0.4% Cellulase<br>0.2% Driselase | TM-3<br>TM-4 | plants<br>plants | -<br>- | RATUSHNYAK, LATYPOV, SAMOYLOV, PIVEN and GLEBA, 1991 |
| *L. hirsutum* | leaf | 0.6% Cellulysin<br>0.1% Macerozyme | CPW | shoots | 25 $\mu mol\ m^{-2}\ s^{-1}$ | MONTAGNO, JOURDAN and BERRY, 1991 |

* Various concentrations used.

The first divisions of *Lycopersicon* protoplasts generally occur within 5 to 7 days with a second division after a further 3 to 5 days (NIEDZ, RUTTER, HANDLEY and SINK, 1985). The importance of culturing protoplasts in the dark at 29°C for the first 21 days was reported by ZAPATA, EVANS, POWER and COCKING (1977). The methods of SHAHIN (1985) greatly enhanced the regeneration of protoplasts and is today used as the model system for the isolation and culture of tomato protoplasts. The recalcitrance of protoplast derived calli of *L. esculentum* cultures to regenerate plants *in vitro* is well known (ZAPATA, EVANS, POWER and COCKING, 1977; MUHLBACH, 1980; ZAPATA and SINK, 1981; ZAPATA, SINK and COCKING, 1981). Responses are cultivar specific and greatly enhanced in *L. peruvianum* (THOMAS and PRATT, 1982a). Since 1977 only a few cultivars of *L. esculentum* were used successfully in the regeneration of shoots from protoplast derived calli. Of these cultivars, cv. Lukullus and the cherry cultivars, were most frequently used in experiments (Table 2.4). In comparative studies of *L. esculentum* and *L. peruvianum* it was shown that protoplasts of the wild species exhibited the higher potential for regeneration *in vitro* (MUHLBACH, 1980).

Cell, tissue and protoplast cultures of tomato are widely used for diverse biological experiments. MEREDITH (1978) obtained stable aluminium-resistant cell clones,

but was unable to regenerate plants from these cells. However, THOMAS and PRATT (1982b) could regenerate paraquat-tolerant plants of *L. esculentum*. Tomato cultures have also been used for other biochemical experiments such as studies on salt-tolerance of callus (TAL and KATZ, 1980) and the sensitivity to the herbicide metribusin (ELLIS, 1978).

As mentioned by MORGAN and COCKING (1982), shoots can easily be induced on cultured leaf tissue and callus containing pre-organised areas. With the loss of these organised areas through subcultures there is a rapid decrease in regeneration capacity. To be successful in employing tissue culture techniques for the genetic improvement of the tomato, the problem of regeneration ability need to be investigated. The inability of protoplasts of the cultivated tomato to regenerate are often overcome by somatic hybridization between *L. esculentum* and *L. peruvianum* protoplasts. In these experiments, protoplasts of *L. peruvianum* with a high morphogenetic potential were fused with protoplasts of the cultivated tomato (O'CONNELL and HANSON, 1985; WIJBRANDI, CAPELLE, HANHART, VAN LOENEN MARTINET-SCHURINGA and KOORNNEEF, 1990; BELLINI, CHUPEAU, GERVAIS, VASTRA and CHUPEAU, 1990; RATUSHNYAK, LATIPOV, SAMOYLOV, PIVEN and GLEBA, 1991). The regeneration capacity was also transferred from *L. peruvianum* to *L. esculentum* by classical breeding

techniques (KOORNNEEF, HANHART and MARTINELLI, 1987). In these experiments segregation data showed that the favourable cell culture traits of *L. peruvianum* are dominant and that regeneration capacity is controlled by two genes.

## 2.6 CYTOKININ-AUXIN RATIOS WITH RESPECT TO ORGANOGENESIS

The view that plant cell division is chemically controlled is not new and it was HABERLANDT (1913) who provided the first evidence for this concept. Many chemical compounds were investigated as potential sources of cell division promoting substances. Particularly rich sources, as evidenced by activity in the tobacco pith bio-assay, were coconut milk, malt extract, yeast extract, extracts of vascular tissue and autoclaved DNA (MILLER, SKOOG, VON SALTZA and STRONG, 1955). The conclusive identification of the first cytokinin was achieved by MILLER, SKOOG, OKAMURA, VON SALTZA and STRONG (1956) when 6-furfurylamino-purine was purified from autoclaved herring sperm DNA. This was however, an artificial cytokinin for it does not occur naturally in plant tissue. The first naturally occurring cytokinin was purified in 1963 by LETHAM from immature kernels of *Zea mays* and identified as 6-(4-hydroxy-3-methylbut-trans-2-benzylamino) purine, more commonly known as zeatin.

**TABLE 2.4** : Cultivars of *L. esculentum* used for protoplast isolations.

| Cultivar | Reference |
|---|---|
| Ailsa | ZAPATA, EVANS, POWER and COCKING, 1977 |
| Nadja | KOBLITZ and KOBLITZ, 1982a |
| Lukullus | KOBLITZ and KOBLITZ, 1982b |
| Lukullus | TEWES, GLUND, WALTHER and REINBOTHE, 1984 |
| Red Cherry<br>Cocktail Cherry<br>VFNT-Cherry<br>VF - 36<br>Manapal<br>Floradade<br>UC - 82<br>Red Ace<br>Roma<br>Beefsteak<br>Improved Pearson<br>Heinz 733<br>Heinz 2152 | SHAHIN, 1985 |
| Lukullus<br>Red Cherry<br>LA 1268<br>L A 1622<br>Sub-Artic Maxi<br>Walter<br>Ventura | NIEDZ, RUTTER, HANDLEY and SINK, 1985 |
| UC 82B<br>Petoseed No. 46 | O'CONNELL and HANSON, 1985 |
| Moneymaker<br>Bellina<br>Sonatine<br>Abunda | TAN, RIETVELD, VAN MARREWIJK and KOOL, 1987 |
| Large Red (Albino mutant) | DERKS, ZELCER and COLIJN-HOOYMANS, 1990 |

Cytokinins have been defined as substances which, in combination with auxin, stimulate cell division in plants and which interact with auxin in determining the direction which differentiation of cells takes (MCGAW, 1987).

In addition to their effects on cell division, cytokinins exhibit a wide range of physiological effects when applied externally to whole plants, plant tissues and plant cells. These include redifferentiation of plant tissues to form organs, cell enlargement and their ability to delay senescence (HORGAN, 1985). The phenomenon of de-differentiation during callus induction is of great interest, since a precise understanding of the changes accompanying this process may go a long way towards answering many questions concerning morphogenesis.

In 1953, MILLER and SKOOG investigated the chemical control of bud formation in tobacco stem segments and it was shown that adenine and IAA, along with phosphate and possibly ribose, participate in a system through which changes in the proportion of the compounds were reflected by changes in growth and formation of organs. The chemical control of morphogenesis and especially the effects of cytokinins on shoot formation can be seen from the work of KARTHA, GAMBORG, SHYLUK and CONSTABEL (1976), DHRUVA, RAMAKRISHIVAN and VAIDYANATHAM (1978) and GUNAY and RAO

(1980) where it was reported that certain combinations of auxins and cytokinins were superior to others and that BA and zeatin could both induce shoots in tomato. Upon investigating the interaction of cytokinin and auxin in cell division in the tobacco pith system, it was found that by manipulation of the auxin:cytokinin ratio, organogenesis could be affected (MURASHIGE, 1977). A high cytokinin:auxin ratio produced shoots whilst a low cytokinin:auxin ratio produced roots. When the ratios were about the same, callus was produced. GEORGE and SHERRINGTON (1984) described a so-called "indirect organogenesis" technique where callus is produced from plant tissue, shoots are induced on this callus tissue by means of a high cytokinin:auxin ratio, the shoots are removed and then rooted in a medium containing either a low cytokinin:auxin ratio or only auxin. Each of these steps may not be discrete or synchronous and the levels of the exogenous hormones may be different. The same principles apply to "direct organogenesis" where the difference is that no callus is developed during proliferation of organized structures.

PALNI, BURCH and HORGAN (1988) studied the effect of NAA concentration on the stability and metabolism of zeatin riboside with the tobacco pith system and found cytokinin stability to be inversely related to the auxin concentration. The results further indicated that this effect may be mediated partly through an enzyme named cytokinin oxidase. Some interesting facts about this enzyme are that zeatin

and ribosylzeatin are substrates of the enzyme, but BA and kinetin are not degraded by it (LETHAM and PALNI, 1983). The enzyme reaction requires oxygen and hence is conveniently termed "cytokinin oxidase". The mechanism of the enzyme reaction is not at all clear. Further, the production of various metabolites of BA such as adenine (Ade), adenosine (Ado), [3G]BA, [9R-MP]BA, [9G]BA and [9R]BA (Table 2.5), found in excised organs of tomato (BAYLEY, VAN STADEN, MALLETT and DREWES, 1989), cannot be explained in terms of the activity of cytokinin oxidase. Hence some plant tissues must contain enzyme systems, distinct from cytokinin oxidase, which cleaves benzyl and furfuryl groups from the $N^6$ position to yield Ade and its derivatives. Two other enzymes involved in BA metabolism could be β-(9-cytokinin-alanine) synthase and cytokinin-7-glucosyltransferase (LETHAM and PALNI, 1983).

The cytokinin:auxin ratio may also be affected by other physiological and morphological factors. The uptake of these cytokinins was found to be limited in the case of kinetin and BA when tested in decapitated roots of tomato (FORSYTH and VAN STADEN, 1987). This showed that the efficiency of a cytokinin could also depend upon the uptake efficiency of the plant material. BAYLEY, VAN STADEN, MALLETT and DREWES (1989) showed that the fate of BA in the tomato is dependent not only on the site of application, but also on the age and

physiological status of the plant. These authors also found that roots and leaves differed significantly in their metabolism of BA.

**TABLE 2.5** : BA and its derivatives (LETHAM, 1978).

| Cytokinin/derivative | Abbreviations |
|---|---|
| base | BA |
| 3-glucoside | [3G]BA |
| 7-glucoside | [7G]BA |
| 9-glucoside | [9G]BA |
| ribonucleoside | [9R]BA |
| 9-ribosylglucoside | [9R-G]BA |
| nucleotide | [9R-MP]BA |
| dinucleotide | [9R-DP]BA |
| trinucleotide | [9R-TP]BA |
| alanine conjugate | [9Ala]BA |
| ortho-OH base | (2OH)BA |
| ortho-OH riboside | (2OH)[9R]BA |
| meta-OH base | (3OH)BA |
| meta-OH riboside | (3OH)[9R]BA |
| ortho-OH-methylthio-9-glucoside | (2OH)[2MeS9G]BA |

## 2.7 CYTOKININ BIOSYNTHESIS

In plants, cytokinins (Table 2.6) are found as tRNA cytokinins as well as free cytokinins. Evidence for the biosynthesis of both types of cytokinins are presented below.

Biosynthesis of tRNA cytokinins are known to occur at the polymer level during post-transcriptional processing (HALL, 1973). The process begins at the point where mevalonic acid pyrophosphate undergoes decarboxylation, dehydration and isomerisation to end with $_{\Delta}^{2}$-isopentenyl pyrophosphate (iPP). The latter then condenses with the relevant adenosine residue in the tRNA to produce the [9R] iP moiety (CHEN and HALL, 1969). An enzyme named $_{\Delta}^{2}$-iPP:tRNA-$_{\Delta}^{2}$-isopentenyl transferase has been partially purified from *Escherichia coli* (BARTZ and SÖLL, 1972). This enzyme could utilize tRNA but not oligoadenylic acids, adenosine or adenosine-5'-monophosphate (AMP) as a substrate. HOLTZ and KLÄMBT (1978) however, purified an enzyme from *Zea mays* which was able to isopentenylate tRNA poly and oligoadenylic acids as well as adenosine. These findings opened the possibility that one enzyme may be responsible for the formation of both tRNA and free cytokinins.

Two mechanisms have been proposed to account for the production of free cytokinins. One involves biosynthesis via tRNA and the other as a direct process.

Since tRNA contains cytokinins, biosynthesis *via* the hydrolysis of tRNA to produce mononucleotides is a possibility (BURROWS, SKOOG and LEONARD, 1971). There are however, several pieces of evidence that suggest it is not a major source of free cytokinin under normal conditions. Plant cultures that require an external supply of cytokinins to sustain growth, contain cytokinins in their tRNA (BURROWS, SKOOG and LEONARD, 1971). BURROWS (1978) also found that certain cytokinins found as free compounds in plants do not occur in the corresponding tRNA. It was also shown that zeatin-like tRNA have the *cis*-configuration

**TABLE 2.6** : Selected examples of naturally occurring cytokinins.

| Name | Abbreviation. | Reference |
|---|---|---|
| Zeatin | Z | VAN STADEN and MENARY, 1976 |
| Zeatin riboside | [9R]Z | LETHAM, 1973 |
| Zeatin-9-β-D-glucoside | [9G]Z | SUMMONS, ENTSCH, PARKER and LETHAM, 1979 |
| Zeatin-7-β-D-glucoside | [7G]Z | EDWARDS and ARMSTRONG, 1981 |
| Lupinic acid | [9 Ala]Z | DUKE, MACLEOD, SUMMONS, LETHAM and PARKER, 1978 |
| Zeatin riboside-5'-monophosphate | [9R-5'P]Z | SONDHEIMER and TZOU, 1971 |
| Zeatin-O-glucoside | (OG)Z | MORRIS, 1977 |
| Zeatin riboside-O-glucoside | (OG)[9R]Z | DUKE, LETHAM, PARKER, MACLEOD and SUMMONS, 1979 |
| dihydrozeatin | (diH)Z | PALNI and HORGAN, 1982 |
| dihydrozeatin riboside | (di)[9R]Z | PALNI and HORGAN, 1982 |
| dihydrozeatin-9-β-D-glucoside | (diH)[9G]Z | WANG, THOMPSON and HORGAN, 1977 |
| dihydrozeatin-7-β-D-glucoside | (diH)[7G]Z | WANG, THOMPSON and HORGAN, 1977 |
| dihydrolupinic acid | (diH)[9 Ala]Z | DUKE, MACLEOD, SUMMONS, LETHAM and PARKER, 1978 |
| dihydrozeatin riboside-5'-monophosphate | (diH)[9R-5'P]Z | SONDHEIMER and TZOU, 1971 |
| dihydrozeatin-O-glucoside | (diHOG)Z | SUMMONS, ENTSCH, LETHAM, GOLLNOW and MACLEOD, 1980 |
| dihydrozeatin riboside-O-glucoside | (diHOG)[9R]Z | DUKE, LETHAM,PARKER, MACLEOD and SUMMONS, 1979 |
| $N^6$ ($_{\Delta}^2$-isopentenyl) adenine | iP | LALOUE, TERRING and GUERN, 1977 |
| $N^6$ ($_{\Delta}^2$-isopentenyl) adenosine | [9R]iP | LALOUE, TERRING and GUERN,1977 |
| $N^6$ ($_{\Delta}^2$-isopentenyl) adenosine-5'-monophosphate | [9R-5'P]iP | LALOUE, TERRING and GUERN, 1977 |
| $N^6$ ($_{\Delta}^2$-isopentenyl) adenine-7-glucoside | [7G]iP | LALOUE, TERRING and GUERN, 1977 |

of the side chain whereas most free cytokinins have the *trans*-side chain configuration (SCOTT and HORGAN, 1984). This was shown in studies with tumor tissues caused by *Agrobacterium tumefaciens*. These findings also indicated that the T-DNA genes are involved in the *de novo* synthesis of cytokinins.

Although a transferase enzyme from the T-DNA of *A. tumefaciens* has been isolated (BARRY, ROGEN, FRALEY and BRAND, 1984), another enzyme has been characterized in *Dictyostelium discoideum* that catalyses the synthesis of [9R-5'P]iP from AMP and $_{\Delta}^{2}$-:iPP (TAYA, TANAKA and NISHIMURA, 1978). CHEN and MELITZ (1979) have also isolated a similar enzyme from a cytokinin-autonomous tobacco callus culture. This enzyme has been called $_{\Delta}^{2}$-isopentenylpyrophosphate : AMP - $_{\Delta}^{2}$ - isopentenyltransferase and catalyses the reaction between 5'-AMP and $_{\Delta}^{2}$ - isopentenylpyrophosphate to form [9R - 5'P]iP. Adenine and adenosine do not act as substrates for this enzyme and the side chain must be the correct isopentenyl isomer and contain a pyrophosphate group. If this enzyme is responsible for the first step in cytokinin biosynthesis two aspects had to be proven, firstly that the product of this reaction [9R-5'P]iP must be stereospecifically hydroxylated to form *trans*-zeatin and secondly, that this hydroxylation must occur very rapidly since [9R-5'P]iP, [9R]iP and iP are very seldom formed in plants (STUCHBURY, PALNI, HORGAN and WAREING,

1979). PALNI and HORGAN (1983) found that $^{14}$C-iP was stereospecifically *trans*-hydroxylated. This suggests that free cytokinin biosynthesis occurs at the nucleotide level (STUCHBURY, PALNI, HORGAN and WAREING,1979).

These results indicate that there is a strong possibility that a *de novo* biosynthetic route exists. There are several reports in the literature of the incorporation of a radioactive label from adenine into cytokinins in plant tissues (PETERSON and MILLER, 1976; STUCHBURY, PALNI, HORGAN and WAREING, 1979; WANG, BEUTELMANN and COVE, 1981). STUCHBURY, PALNI, HORGAN and WAREING (1979) also confirmed that free cytokinin biosynthesis in plants occurs at the nucleotide level. The mechanism of cytokinin biosynthesis in plants appears to involve two routes, one leading to tRNA cytokinins and the other to free cytokinins.

## 2.8 CYTOKININ METABOLISM

When both synthetic and naturally occurring cytokinins are applied to plant tissues they are rapidly metabolized to a variety of different compounds. The various cytokinin compounds are considered separately.

The cytokinin ribosides and their 5'-monophosphates are the most abundant naturally occurring cytokinins in plants (LETHAM, PALNI, TAO, GOLLNOW and BATES, 1983). When $^{14}C$ labelled zeatin is applied externally, the important products produced are [9R]Z and [9R-MP]Z (LETHAM and PALNI, 1983). Where time course studies have been performed it has been found that the nucleotides are the dominant metabolites in the early stages after application and that rapid hydrolysis of the compounds occurs to produce more stable or less biologically active products like N-glucosides and O-glucosides (LETHAM and PALNI, 1983).

CHEN and ECKERT (1977) studied the enzymes responsible for the interconversions of the cytokinin bases, ribosides and ribotides. An adenosine phosphorylase (CHEN and PETSCHOW, 1978), an adenosine kinase (CHEN and ECKERT, 1977) and an adenosine phosphoribosyltransferase (CHEN and ECKERT, 1977) have been characterized. These enzymes exhibited lower affinities for the $N^6$ substituted substrates than for adenine or adenosine. An enzyme which cleaves the isopentenyl sidechain from iP and [9R]iP to yield Ade and Ado has been isolated from kernels of *Zea mays* (WHITTY and HALL, 1974). This enzyme system requires oxygen and is therefore termed "cytokinin oxidase". This enzyme system has also been identified in other sources such as tobacco tissue (PACES and KAMINEK, 1976) and in mosses (GERTHAUSER and BOPP, 1990). In plant

tissues, the cleavage of the sidechains of Z and iP as well as their ribotides are attributable to this enzyme system. It is unclear how analogous degradation of BA and kinetin occur since GERTHAUSER and BOPP (1990) attributes the degradation of these two cytokinins to the existence of a similar "cytokinin oxidase" system. However, the activity of this system is regulated by at least one process. The endogenous levels of auxin had a pronounced effect on the cytokinin activity (PALNI, BURCH and HORGAN, 1988) again emphasizing the importance of enzymatic regulation in the control of the optimum endogenous auxin/cytokinin ratio for the regeneration of shoots.

BA was shown to be converted to its 7- and 9-glucosides by an enzyme system termed cytokinin-7-glucosyl-transferase, isolated from radish cotyledons (ENTSCH and LETHAM, 1979). A β-(9-cytokinin) alanine synthase system has also been isolated from seeds of *Lupinus luteus* which could metabolise zeatin into a ninhydrin reacting compound which was probably lupinic acid (MURAKOSHI, IKEGAMI, OOKAWA, HAGININA and LETHAM, 1977). WASTERNAC, GURANOWSKI, GLUND, TEWES and WALTHER (1985) showed the presence of the four purine metabolizing enzymes, 5'-nucleotide phosphatase, adenine phosphoribosyltransferase, 5'-methylthioadenosine hydrolase and S-adenosylhomocysteine hydrolase in the tomato. These four enzyme systems were

also detected by BURCH and STUCHBURY (1987).

The more unusual metabolites of both naturally occurring and synthetic cytokinins are the variously substituted glucosides. Unlike the ribosyl conjugates, glucosylation is not confined to the α position of the purine ring (LETHAM, PALNI, TAO, GOLLNOW and BATES, 1983). These authors also showed that the 7- and 9-glucosides and side chain O-glucosides are major cytokinins in certain tissues. ENTSCH and LETHAM (1979) isolated two glucosyltransferases from radish cotyledons which could catalyze the formation of 7- and 9-glucosides of benzyladenine using uracyldiphosphate-glucose or tyrosinediphosphate-glucose as glucose source. The ratio of these products, which is [7G]BA and [9G]BA, was different. ENTSCH, LETHAM, PARKER and SUMMONS (1979) have studied these enzymes in relation to a number of synthetic and naturally occurring cytokinins. They found that *cis*-zeatin, *trans*-zeatin and BA gave appreciable quantities of 9-glucosides. There was little difference in the overall rates of N-glucosylation of these cytokinins. The side chain OH-group of zeatin is also a position at which conjugation with glucose occurs (HORGAN, 1985). N-glucosyl conjugation is considered to be important in the regulation of cytokinin activity (LETHAM, PALNI, TAO, GOLLNOW and BATES, 1983). PARKER and LETHAM (1973) found the 7- and 9-glucosides of zeatin to be biologically inactive

and extremely stable in the tissue in which they are formed. PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD (1978) reported that exogenous application of labelled zeatin can lead to the formation of large amounts of O-glucosyl derivatives which remain unmetabolised over long periods. This led to the proposal that O-glucosides may be cytokinin storage forms to yield cytokinins when required (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978). Other evidence for this view has been found in the work of PALMER, HORGAN and WAREING (1981) on endogenous cytokinins of *Phaseolus vulgaris*. Decapitation of these plants lead to a rapid rise in the amount of (diHOG)zeatin in their leaves and upon lateral bud formation the levels of this compound decreased dramatically. VAN STADEN and MALLETT (1988) confirmed that ribosylation and glucosylation are important steps in the metabolism of BA in tomato.

The 9-alanyl conjugation of zeatin and benzyladenine were identified when these cytokinins were exogenously supplied to *Lupinus* (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978). LETHAM and PALNI (1983) found the same with immature apple seeds and derooted *Phaseolus* seedlings. An enzyme called β-(6-allylaminopurine-9-yl) adenine synthase has been characterised from developing seeds of *Lupinus augustifolius* (PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD, 1978). This

enzyme utilises O-acetyl serine as the donor of the alanine residue, but is capable of conjugating a large number of different purine substrates. LETHAM, PALNI, TAO, GOLLNOW and BATES (1983) showed that these compounds are biologically inactive and PARKER, LETHAM, GOLLNOW, SUMMONS, DUKE and MACLEOD (1978) showed them to be stable compounds, which makes the role of these amino-acid conjugates probably similar to that of N-glucosyl cytokinins.

A widespread view regarding the mode of action of plant growth regulators is that the initial event regarding expression of biological activity involves binding of the active substance to a specific receptor molecule.

The first report of a binding factor for cytokinins was by BERRIDGE, RALPH and LETHAM (1970) who described the non-saturable multisite reversible binding of kinetin to ribosomes isolated from leaves of Chinese cabbage. FOX and ERION (1975) discovered a protein with a high specificity and high affinity for cytokinin. A very similar protein has also been isolated from wheat embryos (POLYA and DAVIS, 1978; MOORE, 1979). KEIM, ERION and FOX (1981) found a very high affinity binding factor associated with a mitochondrial fraction from *Phaseolus aureus*. It has, however, not been possible to assign any biological function to this

cytokinin binding.

## 2.9 THE UPTAKE AND METABOLISM OF 6-BENZYLADENINE

The synthetic cytokinin BA is routinely added to tissue culture media and is the most commonly used commercial cytokinin (GEORGE and SHERRINGTON, 1984). Numerous workers have identified metabolites of BA in plant tissue but few have addressed the uptake of BA during organogenesis. LAMPUGNANI, FANTELLI, LONGO, LONGO and ROSSI (1981) studied the uptake of BA by watermelon cotyledons and found the uptake of this cytokinin to be a passive process during a 24 hour period. They also found a positive correlation between the amount of radioactivity in the tissue and the hormonal response. In tuber slices of Jerusalem artichoke it was found that different BA concentrations could induce different rates of BA uptake. At low concentrations a linear uptake was recorded whereas uptake was linear for a shorter period at high concentrations. It was also shown that in this tissue the uptake of BA was an active process (MINOCHA and NISSEN, 1982). BIONDI, CANCIANI and BAGNI (1984) found a rapid uptake of BA in Elm shoots for an initial 24 hour period. Uptake of this cytokinin declined rapidly after five days in culture. In explants of *Picea abies* and *Pinus sylvestris* uptake of BA was again found to be passive and also to be directly

proportional to the exogenous concentration of the cytokinin (VOGELMANN, BORNMAN and NISSEN, 1984). BLAKESLEY (1991) found in *Musa* that after 25 days *in vitro*, 52% of all the available radioactivity (BA) was taken up by the tissue. Similar results was found in cultivars of *Rhododendron*. Shoot cultures of *Gerbera jamesonii* exhibited a linear uptake of BA from the medium during the first 16 days in culture (BLAKESLEY, LENTON and HORGAN, 1991). A similar pattern of BA uptake was reported for apple (NORDSTROM and ELIASSON, 1986) as well as for wild cherry (LABEL, MALDINEY, SOSSOUNTZOV, CORNU and MIGINIAC, 1988). During 1992 studies on specific characteristics of BA uptake were expanded. AUER, LALOUE, COHEN and COOKE (1992) characterized BA uptake during the key stages of shoot induction and development in leaf explants of *Petunia*. In these experiments it was shown that exposure to BA for 6, 8 and 10 days induced shoot formation. Leaf explants produced the optimum amount of shoots after 10 days of exposure to BA. The rate of BA uptake was also studied in a range of species by BLAKESLEY and CONSTANTINE (1992) and it was found that the rate of uptake varied between the species. In leaf discs of *L. esculentum* cv. Rodade it was found that a minimum exposure period of 7 days was necessary for the induction of adventitious shoots and that maximum induction could be achieved after 35 days of exposure to BA (DE VILLIERS, JANSE VAN VUUREN, FERREIRA and VAN STADEN, 1993).

The functional significance of BA metabolites is somewhat obscure. The following functions have been attributed to such metabolites:

1. Active forms
2. Translocatory forms
3. Storage forms
4. Detoxification forms
5. Deactivation forms
6. Inactivation products

In commercial practice, BA is often used for shoot multiplication in a wide range of species, because it is highly active and relatively inexpensive (BLAKESLEY, LENTON and HORGAN, 1991). Several workers have identified metabolites of BA in plant tissue (LETHAM and PALNI, 1983). It is often found that different species show differences in the metabolism of BA and the true function of the metabolites are therefore probably quite different.

Tomato roots showed the production of [9R]BA, [9G]BA, a conjugated derivative of [9R]BA as well as a possible phosphorylated derivative (FORSYTH and VAN STADEN, 1987). No unmetabolized BA was detected in the tissue. NANDI, PALNI, LETHAM and WONG (1989) identified BA its riboside and corresponding

nucleotide as endogenous cytokinins in crown gall tumours of tomato. These results were confirmed by NANDI, LETHAM, PALNI, WONG and SUMMONS (1989). A great deal of interconversion between BA, [9R]BA and [9R-MP]BA were found in tomato shoots (VAN STADEN and BAYLEY, 1991). It was however, unclear whether [9R-MP]BA were formed directly from BA or whether [9R]BA served as an intermediate metabolite. BLAKESLEY, LENTON and HORGAN (1991) isolated [9R-G]BA from shoot cultures of *Gerbera jamesonii*. Several glucosides together with [9R]BA were also isolated. It is therefore clear that BA metabolism varies considerably between species (BLAKESLEY and CONSTANTINE, 1992). Also, when comparing activities of different enzyme systems it becomes clear that the metabolism of BA is not only influenced by the activity or presence of these enzyme systems, but that metabolism may also be limited by competition for these enzymes (BURCH and STUCHBURY, 1987).

In tomato, degradative metabolism of BA is primarily in the roots, but it is not confined to this organ. Small amounts of [9R-MP]BA were found in roots (BAYLEY , VAN STADEN, MALLET and DREWES, 1989). They also showed that these conjugates are the major translocatory forms of BA. VAN STADEN and BAYLEY (1990) showed in *Phaseolus vulgaris* that BA are more extensively metabolised by roots than by stem or leaf tissue.

The major forms of BA metabolism in tomato appear to be ribosylation (BAYLEY, VAN STADEN, MALLETT and DREWES, 1989). In *Phaseolus* most of the BA absorbed was converted to ribosylbenzyladenine whereas degradative metabolism by side-chain cleavage and conjugation by glucosylation were minor events (VAN STADEN and BAYLEY, 1990). In *Petunia* leaf explants, glucosylation of BA is a major metabolic pathway (AUER, LALOUE, COHEN and COOKE, 1992). The fate of BA in the tomato is thus dependant not only on the site of application and movement of the cytokinins into a particular organ, but also on the age and physiological status of the plant tissue (BAYLEY, VAN STADEN, MALLETT, and DREWES, 1989).

LETHAM, PALNI, TAO, GOLLNOW and BATES (1983) showed with bioassays that the 7- and 9-glucosides of BA had lower activities than BA itself, whereas [3G]BA had a higher activity than BA. Lupinic acid on the other hand is weakly active. The different activities could be attributed to the ability of tissue to cleave the 3-glucoside moiety to release BA. BLAKESLEY (1991) showed in shoot cultures of *Musa* that BA is converted to [9G]BA which acts as a storage product. BLAKESLEY, LENTON and HORGAN (1991) suggested that BA or [9R]BA were associated with shoot proliferation whereas, [9R]BA is the translocatable form of BA. Other metabolites such as [3G]BA, [9G]BA and [9R-G]BA might be

associated with either storage or detoxification. In *Petunia* leaf explants BA ribotides may serve as short term storage compounds or alternatively may constitute the active form of cytokinins responsible for shoot induction (AUER, LALOUE, COHEN and COOKE, 1992). Studies on soybean callus (VAN STADEN and BAYLEY, 1991) showed that the free base of BA is the active form of the cytokinin and that [3G]BA constitutes a storage form. It was also concluded that the 7- and 9-glucosides of BA could act as a detoxification/inactivation compounds.

## 2.10 METHODS OF CYTOKININ ANALYSIS

The very small amounts of cytokinins found in most plant tissues create considerable difficulties for the isolation and identification of these compounds (HORGAN, 1985). A major problem is the isolation of such small amounts of cytokinins to a sufficiently high degree of purity for analysis. A discussion of these techniques follows.

### 2.10.1 Extraction and purification of cytokinins

Methods for the extraction of cytokinins from plant material usually include solvent partitioning, ion exchange chromatography as well as paper and thin layer

chromatography (TLC) (HORGAN, 1987).

It was MILLER (1965) who showed the existence of zeatin and its derivatives by extracting these compounds with either cold or boiling ethanol from frozen kernels of *Zea mays*. Since that time, very little has changed and cytokinins such as kinetin, BA, zeatin and all their derivatives have been extracted from plant material by means of ethanol (VAN STADEN and FORSYTH, 1985; FORSYTH and VAN STADEN, 1987; VAN STADEN and MALLETT, 1988; PALNI, BURCH and HORGAN, 1988; BAYLEY, VAN STADEN, MALLETT and DREWES, 1989; VAN STADEN and BAYLEY, 1990). After extraction the dissolved substances are concentrated by means of vacuum evaporation, resuspended in methanol and filtered through a 0.22 μm filter (VAN STADEN and MALLETT, 1988). This method seems to be simple but effective since other workers such as PALNI, BURCH and HORGAN (1988) achieved no better results with more complicated methods of extraction.

### 2.10.2 Bioassays

It should be noted at this point that the primary detection of any novel plant hormone is dependent on bioassays, especially when studying the content of novel

plant extracts with regard to gibberellins and cytokinins (HORGAN, 1987). In the case of cytokinins, which are promoters of cell division in callus cultures, the tobacco pith bioassay (MURASHIGE and SKOOG, 1962) or the soybean callus bioassay (MILLER, 1965) is employed to reveal the presence of cytokinins in a plant extract. However, these bioassays require 21 days or more to provide results and the *Amaranthus* betacyanin bioassay (BIDDINGTON and THOMAS, 1973) is therefore more frequently used for the monitoring of cytokinin activity. A new bioassay for the detection of auxins and cytokinins was recently developed by BOERJAN, GENETELLO, VAN MONTAGU and INZE (1992) based on the expression of a chimeric gene consisting of upstream sequences of the *Agrobacterium tumefaciens* gene 5, coupled to the coding sequence of the β-glucoronidase enzyme in mesophyll protoplasts of tobacco. The expression of the gene is induced by auxin or cytokinin allowing the reaction to be visualized by ultra violet light.

### 2.10.3 Immunological methods

There has been much interest recently in the application of immunoassays for the detection and quantification of growth regulators. These assays are based on the ability of animals to produce antibodies which bind to specific compounds named

antigens. This technique was first applied to plant hormones in an assay measuring the inactivation of hormone-labeled bacteriophage by antibodies raised against the hormone (FUCHS, HAIMOVICH and FUCHS, 1971). These methods has already been implimented for the detection and quantitation of IAA, abscisic acid (ABA), cytokinins and gibberellins (PENCE and CARUSO, 1987). Enzyme linked immunoassays (ELISA) are usually used for the analysis of cytokinins. Antigens for the production of antibodies to cytokinins and enzyme tracers to cytokinin-ELISA are synthesised using cytokinin ribosides by a periodate reaction which breaks the sugar ring and link amino groups of the carrier protein to the resulting aldehyde (VOLD and LEONARD, 1981). Antibodies have also been used for immunoaffinity purification of growth-regulators and localization of hormones (PENCE and CARUSO, 1987). Radioimmunoassays combined with HPLC is also a very popular method of analysis (MACDONALD, AKIYOSHI and MORRIS, 1987; MORRIS, JAMESON, LALOUE and MORRIS, 1991).

### 2.10.4 High performance liquid chromatography (HPLC)

HPLC is distinguished from other more traditional forms of chromatography by its high efficiency and resolution and speed of separation (HORGAN, 1987). In assessing HPLC methods for plant analysis it is important to appreciate the

presence of two often conflicting factors. Firstly, the need to separate the hormone from the compounds present in the extract and secondly, the ability of the system to separate closely related growth regulators (HORGAN, 1987). For plant hormone HPLC the most frequently used stationary phase is octadecylsilane (ODS) which is covalently bonded to microparticulate or microspherical silica as described by HORGAN and KRAMERS (1979). They also showed that solvents should be binary mixtures or gradients of water and the lower alcohols such as methanol, ethanol and butanol or acetonitrile.

Reversed phase HPLC is at its best when the compounds being analyzed are un-ionised and neutral buffers are preferred to suppress the ionisation of basic compounds such as cytokinins. HORGAN (1987) states the value of reverse phase HPLC is the enormous range of compound polarities that can be accommodated on a single stationary phase when gradient elution is used. Under these conditions polar compounds will elute before non-polar compounds and therefore polar plant hormone conjugates and free cytokinins can be fractionated in a single step (VAN STADEN and MALLETT, 1988).

## 2.11 SUMMARY

A wealth of information is available on the tomato genome (ZAMIR and TANKSLEY, 1988) and the importance of the tomato cultivar Rodade is quite clear (BOSCH and OLIVIER, 1985). The problem lies however, with the regeneration of adventitious shoots from different tissue types of some tomato cultivars since it is an important step towards the improvement of these cultivars through biotechnology. It has been known for quite some time that *L. peruvianum* produce much better results *in vitro* than *L. esculentum* (DE LANGHE and DE BRUIJNE, 1976) and to compare the two species in order to improve tissue culture techniques is nothing new.

In many studies BA and zeatin were found to be the most effective for shoot regeneration (GUNAY and RAO, 1980), but other factors such as the existence of attached organized tissue (HERMAN and HAAS, 1978), explant type, subculture interval, environmental conditions and exposure period also had an effect on regeneration (KABURU M'RIBU and VEILLEUX, 1990; COMPTON and VEILLEUX, 1991; DE VILLIERS, JANSE VAN VUUREN, FERREIRA and VAN STADEN, 1993). The difficulty of regenerating shoots from tomato tissue was also demonstrated by the efforts of VNUCHKOVA (1977) who tested 150 variants of

media for regeneration in tomato. The morphogenetic potential of tomato protoplasts is so low that somatic hybridization is often used to improve the regeneration potential (RATUSHNYAK, LATYPOV, SAMOYLOV, PIVEN and GLEBA, 1991).

The important role played by cytokinins as well as by the cytokinin:auxin ratio is clear (GUNAY and RAO, 1980). It is also evident that auxin has a definite effect on the stability of endogenous cytokinin activity (PALNI, BURCH and HORGAN, 1988) and that this ratio is affected by the age and physiological status of the tissue (BAYLEY, VAN STADEN, MALLETT and DREWES, 1989). Benzyladenine is an important cytokinin since it is often used in commercial tissue culture systems (GEORGE and SHERRINGTON, 1984) but as in the case of many other cytokinins, differences in the uptake and metabolism of BA exists (BLAKESLEY, 1991; BLAKESLEY, LENTON and HORGAN, 1991; VAN STADEN and BAYLEY, 1991). This cytokinin is however commonly used today in the regeneration of adventitious shoots from various cultivars of *L. esculentum*.

Plant biotechnology has come a long way since its humble beginnings in 1902 when HABERLANDT conducted experiments with various cell types *in vitro* and has become an inseparable part of practical plant breeding. In the case of the

cultivated tomato (*Lycopersicon esculentum* Mill.) this aspect is of special importance since the tomato is very amenable to biotechnological approaches and a plethora of knowledge is available on the genome of this crop. The tomato cultivar Rodade is of international importance because if its resistance to bacterial wilt under tropical conditions and is therefore an excellent source of breeding material to improve the cultivated tomato. However, this cultivar is recalcitrant in its ability to regenerate adventitious shoots from callus tissue *in vitro*. As described in the literature, 6-benzyladenine (BA) plays an important role during organogenesis in the tomato, but no data is available concerning the *in vitro* response to BA as well as the uptake and metabolism of this cytokinin in callus tissue of the tomato cultivar Rodade. Since an investigation of these aspects would go a long way towards understanding the recalcitrance of callus tissue of this cultivar to regenerate shoots *in vitro* this study is of major importance to all breeding programmes that will employ cultivar Rodade as a new genetic source for improving resistance against bacterial wilt. This study will also contribute towards a better understanding of the metabolism of BA in tomato tissues as well as the fate of BA and its metabolites during organogenesis in the cultivated tomato under tissue culture conditions.

# CHAPTER 3

# THE REGENERATION OF ADVENTITIOUS SHOOTS.

## 3.1 INTRODUCTION

Organs such as shoots, leaves and flowers can frequently be induced to form adventitiously on cultured plant tissues (GEORGE and SHERRINGTON, 1984). Two different methods of morphogenesis were described by HICKS (1980), the first being a "direct method" of morphogenesis where meristems arise from differentiated cells without the proliferation of undifferentiated tissue. The second being an "indirect method" of morphogenesis where meristems occur from unspecialised and unorganised cells of callus tissue or suspension cultures.

Attempts to assess the morphogenetic potential of leaves of different strains of the cultivated tomato were reported by PADMANABHAN, PADDOCK and SHARP (1974). They reported on a combination of IAA and kinetin that could induce shoots in leaf callus of tomatoes. KARTHA, GAMBORG, SHYLUK and

CONSTABEL (1976) worked with leaf explants of *L. esculentum* cv. Starfire and found that BA in combination with IAA was superior with respect to plantlet regeneration when used in combination with NAA. Zeatin in combination with IAA was the only other hormone combination able to compete with BA and IAA and large numbers of plantlets were regenerated. Fifteen tomato varieties were screened by BEHKI and LESLEY (1976) for a morphogenetic response using NAA and BA. Shoots were regenerated from 12 varieties. In some cases it was impossible to distinguish between NAA and IAA as auxin supplement as far as efficiency was concerned (KARTHA, CHAMPOUX and PAHL, 1977). TAL, DEHAN and HEIKIN (1977) showed that *L. peruvianum* had a much higher morphogenetic potential than *L. esculentum* with respect to root and shoot formation. They also concluded that shoot formation was optimal with the use of kinetin and IAA in tomatoes. A total of 150 different variants of media were screened by VNUCHKOVA (1977) for their ability to induce shoots in leaf callus of tomato. It was found that IAA in combination with kinetin gave the best results. DHRUVA, RAMAKRISHIVAN and VAIDYANATHAM (1978) found it difficult to distinguish between BA or zeatin in combination with IAA since both combinations induced shoots from leaf callus of *L. esculentum*. The importance of the genotype was stressed by MEREDITH (1979) who showed that a specific cell line could produce more adventitious shoots than others. Similar results were found

by GUNAY and RAO (1980).

It is clear that great variability exists in the morphogenetic potential of the tomato (MEREDITH, 1979) and that many conflicting reports of regeneration potential can be found. UDDIN, BERRY and BISGES (1988) worked with 20 processing tomato cultivars and found a combination of kinetin and IAA to be as effective as zeatin and IAA and yet there are numerous other reports showing explicitly that some treatments are better than others. Even within a single cultivar, differences can be found in the different explant sources such as stem, leaf, cotyledon and hypocotyl tissue (MEREDITH, 1979).

Assessment of the morphogenetic potential of leaf and callus tissue of *L. esculentum* and *L. peruvianum* is an important preliminary step in the investigation of BA metabolism in tomatoes since the appearance of adventitious shoots is yet another indicator of cytokinin action. The optimal media for the initiation of adventitious shoots from both leaf and callus tissue of the two species were therefore determined. Only BA and IAA were tested for shoot induction since the literature does not indicate that better results may be obtained using other cytokinin:auxin combinations.

## 3.2 MATERIALS AND METHODS

### 3.2.1 The production of leaf material

Seeds of *Lycopersicon esculentum* cultivar Rodade and *Lycopersicon peruvianum* were surface sterililzed for 6 minutes in 3% (w/v) NaOCl followed by 2 minutes in 95% (v/v) ethanol whereafter they were rinsed twice in sterile double distilled water (DD $H_2O$). The seeds were inoculated on a modified MURASHIGE and SKOOG (MS) nutrient medium (MURASHIGE and SKOOG, 1962) supplemented with myo-inositol (100 mg $dm^{-3}$), sucrose (30 g $dm^{-3}$), caseien hydrolysate (2 g $dm^{-3}$) and agar (8 g $dm^{-3}$). No growth regulators were added to the medium (MSO). The pH was set at 5.8 with 1 M KOH. The cultures were incubated at 25°C and a 16h/8h light/dark regime provided by cool white fluorescent lights with an intensity of 25 μmol $m^{-2}$ $s^{-1}$.

After three weeks, the seedlings were decapitated above the cotyledons and the upper part of the seedling transferred to a fresh medium of the same composition. The remaining hypocotyl segments were used to initiate callus.

After another week the first fully expanded leaves were used to provide leaf discs

that at this stage were four weeks old. To obtain older leaf material the plantlets were decapitated weekly and the upper part of the plantlets transferred to a fresh medium as decribed above.

### 3.2.2 The production of callus

Hypocotyls of both *L. esculentum* and *L. peruvianum* were cut aseptically into 10 mm segments and inoculated onto a modified MS medium supplemented with myo-inositol (100 mg $dm^{-3}$), sucrose (30 g $dm^{-3}$), caseien hydrolysate (2 g $dm^{-3}$) 2,4-dichlorophenoxy acetic acid (2,4-D) (2.0 mg $dm^{-3}$), kinetin (KIN) (0.25 mg $dm^{-3}$) and agar (8 g $dm^{-3}$). The pH was set at 5.8 with 1 M KOH prior to autoclaving. The cultures were incubated at 25°C and a 16h/8h light/dark regime under cool white fluorescent lights with an intensity of 25 μmol $m^{-2}$ $s^{-1}$. For maintenance purposes the calli were subcultured onto a fresh medium of the same composition every two weeks.

Before using the callus in any experiments it was transferred to a MS medium without growth regulators for a period of two weeks. During this time the calli turned green. This holding time also helped to eliminate the inhibitory effects of 2,4-D on regeneration.

### 3.2.3 Preparation of material for factorial treatments

Four-week-old leaves were removed from sterile plantlets, the proximal and distal ends were removed aseptically with a sterile scalpel. The remaining part of the leaf was then cut in half and the two pieces were transferred to the appropriate medium.

In experiments where callus was used, green callus were cut into ±5x5x5 mm blocks and transferred to the appropriate medium.

### 3.2.4 Factorial experiments with BA and IAA

Leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were used in the factorial experiments with BA and IAA in order to determine the optimal concentrations and combinations of these growth regulators for the initiation of adventitious shoots.

The basic medium consisted of a modified MS medium supplemented with myo-inositol (100 mg $dm^{-3}$), sucrose (30 g $dm^{-3}$), caseien hydrolysate (2 g $dm^{-3}$) and agar (8 g $dm^{-3}$). A combination of growth regulators as outlined in table 3.1 were used.

**TABLE 3.1** : Numbers used for the different combinations of BA and IAA concentrations used in the factorial treatments

| BA ($mg\ dm^{-3}$) | IAA ($mg\ dm^{-3}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0.0 | 0.1 | 1.0 | 2.0 | 3.0 | 4.0 |
| 0.0 | 1 | 2 | 3 | 4 | 5 | 6 |
| 0.1 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1.0 | 13 | 14 | 15 | 16 | 17 | 18 |
| 2.0 | 19 | 20 | 21 | 22 | 23 | 24 |
| 3.0 | 25 | 26 | 27 | 28 | 29 | 30 |
| 4.0 | 31 | 32 | 33 | 34 | 35 | 36 |

A second factorial experiment was designed with BA and IAA to include the higher range of growth regulators in order to determine the optimal concentration of growth regulators for the initiation of adventitious shoots.

The basic medium had the same composition as described above. A combination of growth regulators as outlined in table 3.2 were used.

**TABLE 3.2 :** Numbers used for the different combinations of BA and IAA concentrations used in the additional factorial treatments

| BA ($mg\ dm^{-3}$) | IAA ($mg\ dm^{-3}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.0 | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 |
| 0.0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 1.0 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 2.0 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| 3.0 | 22 | 23 | 24 | 25 | 26 | 27 | 28 |
| 4.0 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
| 5.0 | 36 | 37 | 38 | 39 | 40 | 41 | 42 |
| 6.0 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |

The pH of each medium in both experiments was adjusted to 5.8 with 1 M KOH prior to autoclaving. Each medium was dispensed into 20 glass specimen tubes (100 mm x 20 mm id.). Each specimen tube contained 10 $cm^3$ medium. The specimen tubes were covered with transparent plastic lids and were sterilized in an autoclave at 125°C for 20 minutes.

### 3.2.5 Factorial experiments with zeatin and IAA

Leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were used in the factorial experiments with zeatin and IAA in order to determine the optimal concentration and combination of these growth regulators for the initiation of adventious shoots.

The basic medium was the same as described above (Paragraph 3.2.4) except for the combination of growth regulators which were used as outlined in table 3.3.

**TABLE 3.3** : Numbers used for the different combinations of zeatin and IAA concentrations used in the factorial treatments.

| Zeatin ($mg\ dm^{-3}$) | IAA ($mg\ dm^{-3}$) 0.0 | 0.1 | 1.0 | 2.0 | 3.0 | 4.0 |
|---|---|---|---|---|---|---|
| 0.0 | 1 | 2 | 3 | 4 | 5 | 6 |
| 0.1 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1.0 | 13 | 14 | 15 | 16 | 17 | 18 |
| 2.0 | 19 | 20 | 21 | 22 | 23 | 24 |
| 3.0 | 25 | 26 | 27 | 28 | 29 | 30 |
| 4.0 | 31 | 32 | 33 | 34 | 35 | 36 |

The culture vessels used was as described above (Paragraph 3.2.4).

### 3.2.6 Culture conditions

All cultures were incubated at 25°C and a 16h/8h light/dark regime under cool white fluorescent lights with an intensity of 25 $\mu mol\ m^{-2}\ s^{-1}$. A single explant was placed onto the 10 $cm^3$ medium in a specimen tube.

### 3.2.7 Data collection and analysis

Twenty replicates were used in each treatment.The average number of adventitious shoots was recorded and the least significant difference (LSD) ($p<0.01$) calculated for each experiment.

## 3.3 RESULTS

### 3.3.1 The effect of BA and IAA on shoot formation

#### 3.3.1.1 Shoot induction on leaf tissue of *L. esculentum*

In the experiments with 36 factorial combinations of BA and IAA, it was found that adventitious shoots were induced in the range from 2.0 to 4.0 mg $dm^{-3}$ BA. The average number of shoots increased with increasing BA concentration (Figure 3.1). IAA concentration also had an effect on shoot induction. At 2.0 mg $dm^{-3}$ BA an IAA concentration of at least 3.0 mg $dm^{-3}$ was necessary to induce the production of adventitious shoots. The number of adventitious shoots increased with increasing IAA concentration at BA concentrations of 3.0 and 4.0 mg $dm^{-3}$. At BA concentrations of 3.0 and 4.0 mg $dm^{-3}$ it was also found that

no IAA was necessary to induce adventitious shoots. The average number of adventitious shoots in these treatments were however, significantly lower than in the treatments where IAA was included.

In the experiment with 49 factorial combinations of BA and IAA (Figure 3.2) the average number of shoots increased when the BA concentration was increased from 2.0 to 4.0 mg $dm^{-3}$, whereafter the average number of shoots formed, declined. A significant increase in the number of shoots was detected with a BA concentration of 4.0 mg $dm^{-3}$. BA concentrations higher than 4.0 mg $dm^{-3}$ had a negative effect on shoot regeneration in tomato leaf tissue. In this experiment it was also found that the average number of adventitious shoots increased with increasing IAA concentration to a maximum level of 4.0 mg $dm^{-3}$. IAA concentrations higher than 4.0 mg $dm^{-3}$ caused a notable decrease in the number of shoots produced.

In both experiments with *L. esculentum* leaf tissue it was found that the best treatment with respect to the regeneration of adventitious shoots was at a BA concentration of 4.0 mg $dm^{-3}$ and an IAA concentration of 4.0 mg $dm^{-3}$. Treatments with both BA and IAA above and below these concentrations were below optimal.

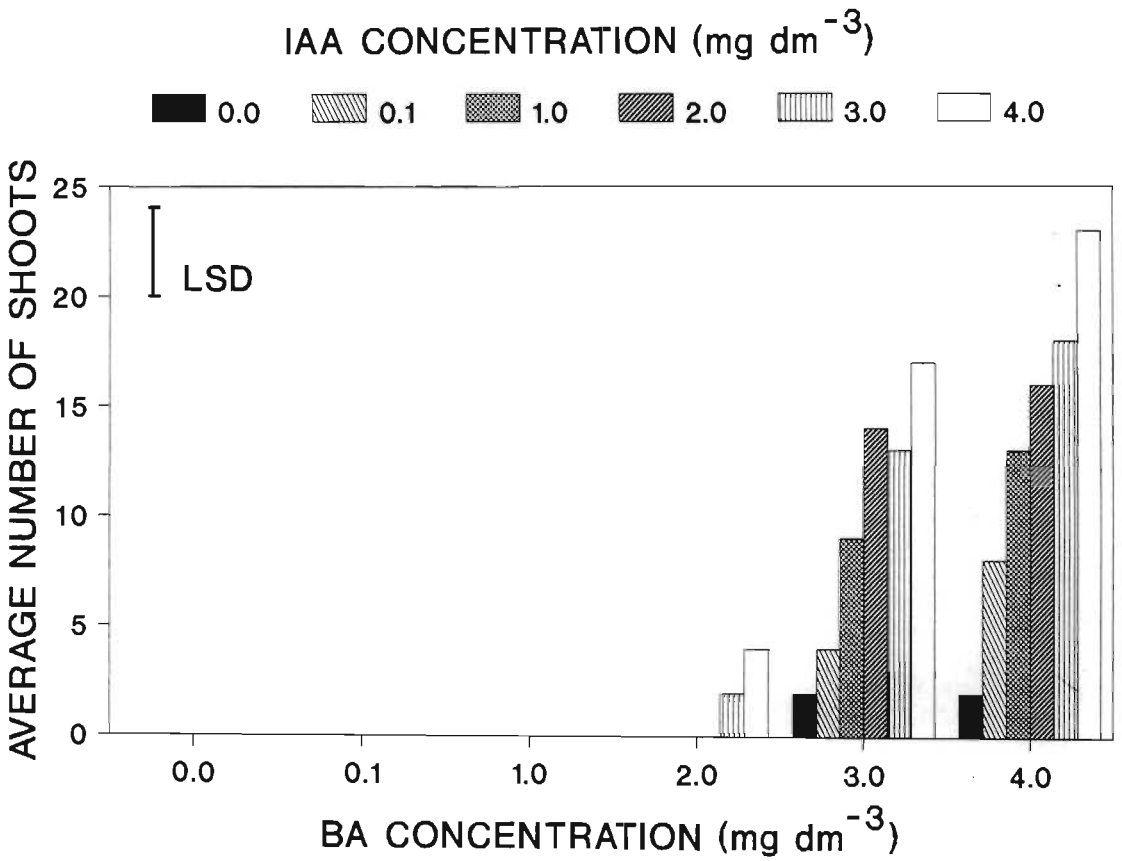


**FIGURE 3.1**: The average number of adventitious shoots formed per leaf explant of *L. esculentum* using 36 factorial combinations of BA and IAA.

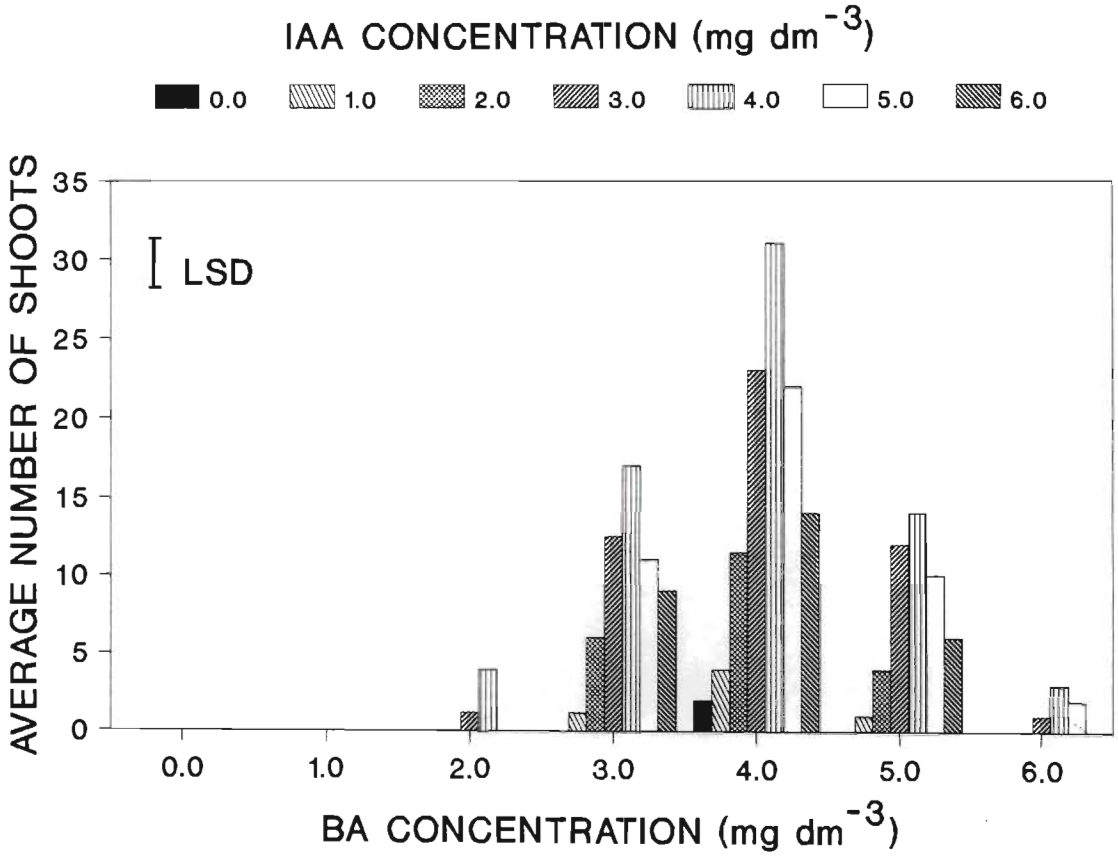


**FIGURE 3.2** : The average number of adventitious shoots formed per leaf explant of *L. esculentum* using 49 factorial combinations of BA and IAA.

### 3.3.1.2 Shoot induction on callus tissue of *L. esculentum*

No shoot regeneration was found with any combination of BA and IAA using callus tissue of *L. esculentum*.

### 3.3.1.3 Shoot induction on leaf tissue of *L. peruvianum*

In the experiment with 36 factorial combinations of BA and IAA the first signs of shoot regeneration was found at a BA concentration of 2.0 mg $dm^{-3}$ (Figure 3.3). An increase in the average number of shoots was found with increasing BA concentration in the range from 2.0 to 4.0 mg $dm^{-3}$. At both 3.0 and 4.0 mg $dm^{-3}$ BA the average number of shoots increased with increasing IAA concentration. A maximum response was achieved with 1.0 mg $dm^{-3}$ IAA. Concentrations of IAA above 1.0 mg $dm^{-3}$ caused a decrease in the average number of adventitious shoots produced. The optimal treatment with respect to shoot formation was induced with a combination of 4.0 mg $dm^{-3}$ BA and 1.0 mg $dm^{-3}$ IAA.

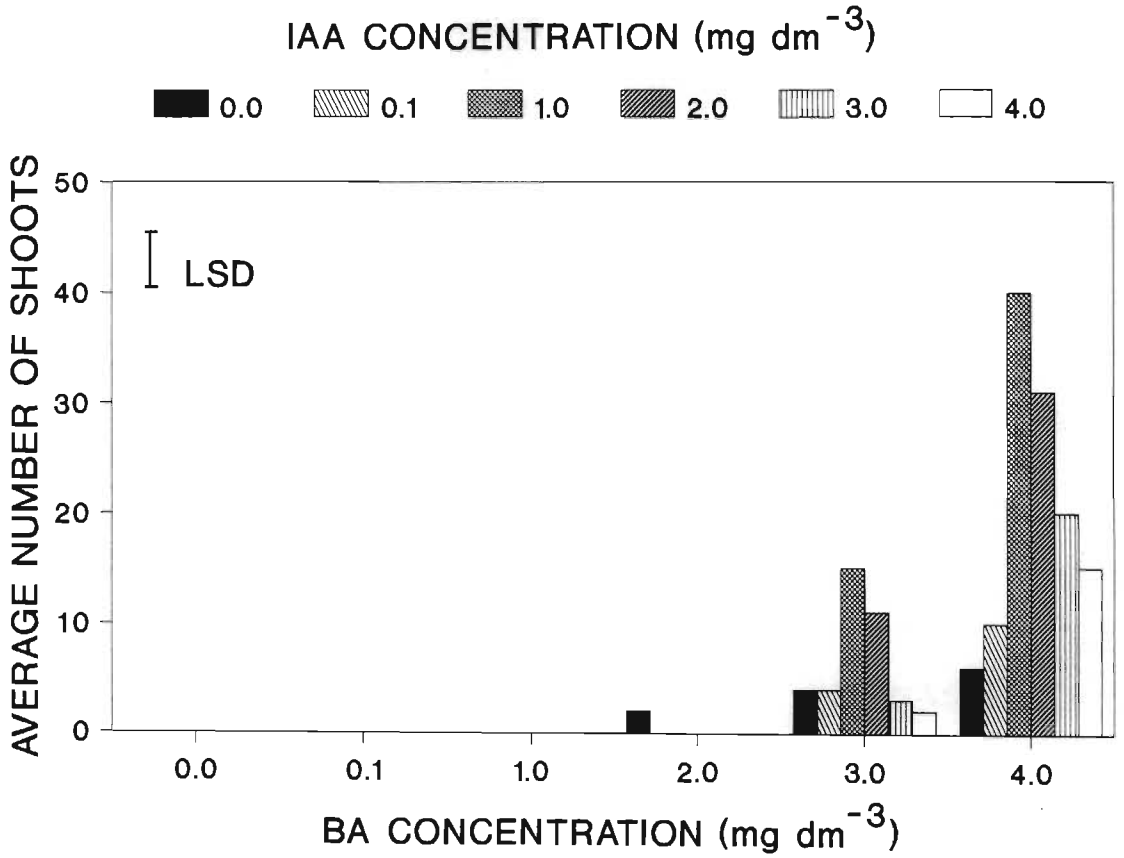


**FIGURE 3.3** : The average number of adventitious shoots formed per leaf explant of *L. peruvianum* using 36 factorial combinations of BA and IAA.

In the 49 factorial it was found that the average number of shoots increased with increasing BA concentration from 3.0 to 4.0 mg $dm^{-3}$. At BA concentrations from 5.0 to 6.0 mg $dm^{-3}$ a decline in the average number of adventitious shoots was recorded (Figure 3.4). Significant differences were found between the shooting response caused by 3.0, 4.0 and 5.0 mg $dm^{-3}$ BA. At IAA concentrations from 0.0 to 1.0 mg $dm^{-3}$ an increasing average number of adventitious shoots were recorded with the treatment at 1.0 mg $dm^{-3}$ IAA being significantly higher than any other treatment at a specific BA concentration. IAA concentrations above 1.0 mg $dm^{-3}$ showed a decrease in shoot production.

In both experiments with leaf tissue of *L. peruvianum* the best combination with respect to the regeneration of adventitious shoots was a BA concentration of 4.0 mg $dm^{-3}$ and an IAA concentration of 1.0 mg $dm^{-3}$. Treatments with BA and IAA both below and above this treatment gave decreased shooting responses.

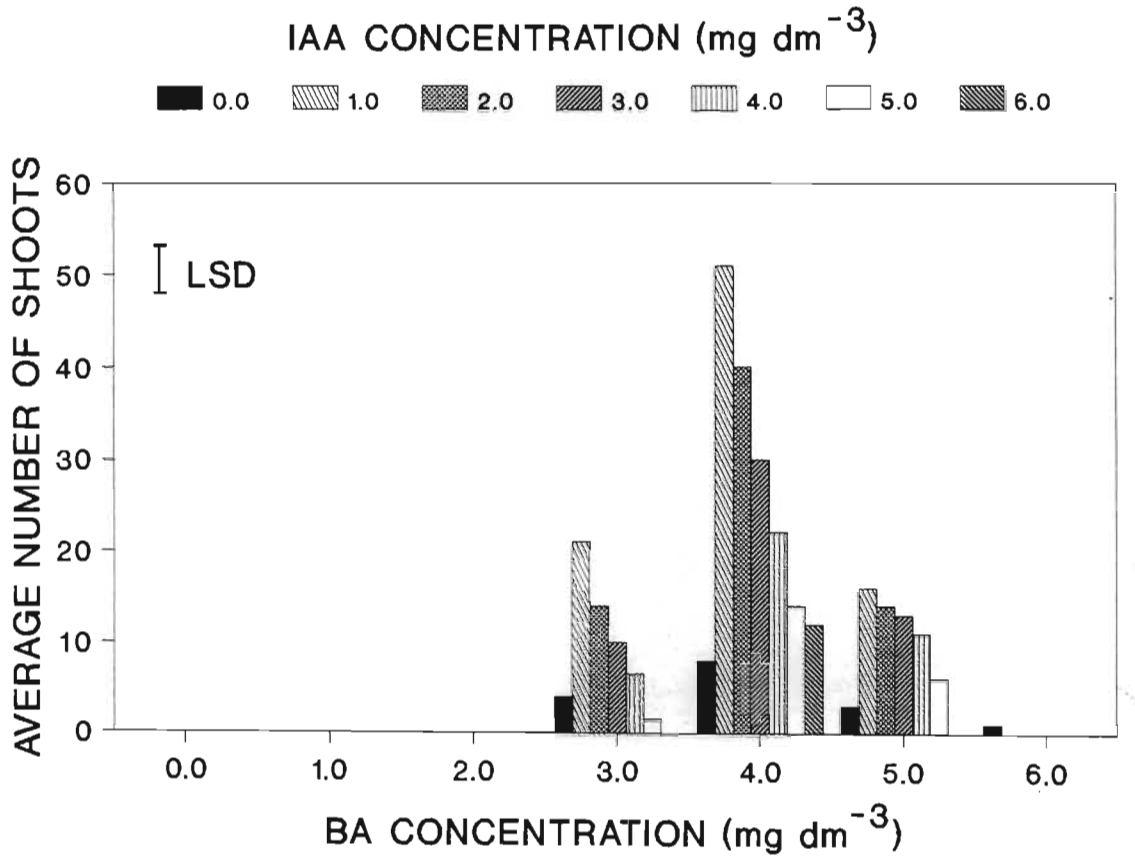


**FIGURE 3.4** : The average number of adventitious shoots formed per leaf explant of *L. peruvianum* using 49 factorial combinations of BA and IAA.

### 3.3.1.4 Shoot induction on callus tissue of *L. peruvianum*

In the experiment with the 36 factorial combinations of BA and IAA it was found that only 4.0 mg $dm^{-3}$ BA induced the regeneration of adventitious shoots (Figure 3.5). The most adventitious shoots were recorded at 0.1 mg $dm^{-3}$ IAA. All IAA concentrations higher than 0.1 mg $dm^{-3}$ caused a significant decline in the average number of adventitious shoots.

In the 49 factorial increasing concentrations of BA induced increasing numbers of adventitious shoots in the range from 3.0 to 4.0 mg $dm^{-3}$. At 5.0 mg $dm^{-3}$ BA the average number of shoots declined, with no shoot induction at 6.0 mg $dm^{-3}$ (Figure 3.6). With 4.0 mg $dm^{-3}$ BA, IAA concentrations from 0.0 to 1.0 mg $dm^{-3}$ caused an increasing number of adventitious shoots with increasing IAA concentration. IAA concentrations between 2.0 to 6.0 mg $dm^{-3}$ did however cause a decline in the average number of adventitious shoots.

The two experiments with callus tissue of *L. peruvianum* showed that the optimal BA concentration for the regeneration of adventitious shoots is at 4.0 mg $dm^{-3}$, whereas the optimal IAA concentration is at 0.1 mg $dm^{-3}$ (Figure 3.6). Treatments with BA and IAA concentrations both above and below this treatment produced significantly less shoots.

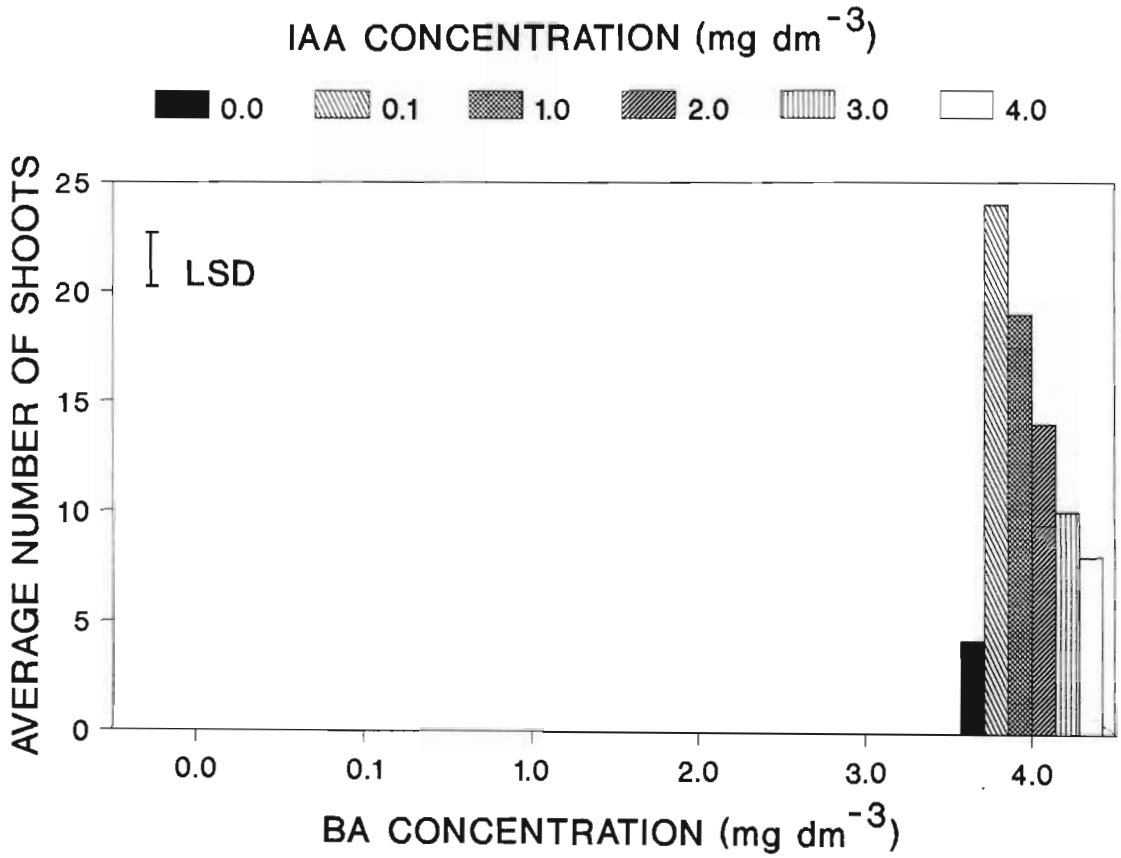


**FIGURE 3.5** : The average number of adventitious shoots formed per callus explant of *L. peruvianum* using 36 factorial combinations of BA and IAA.

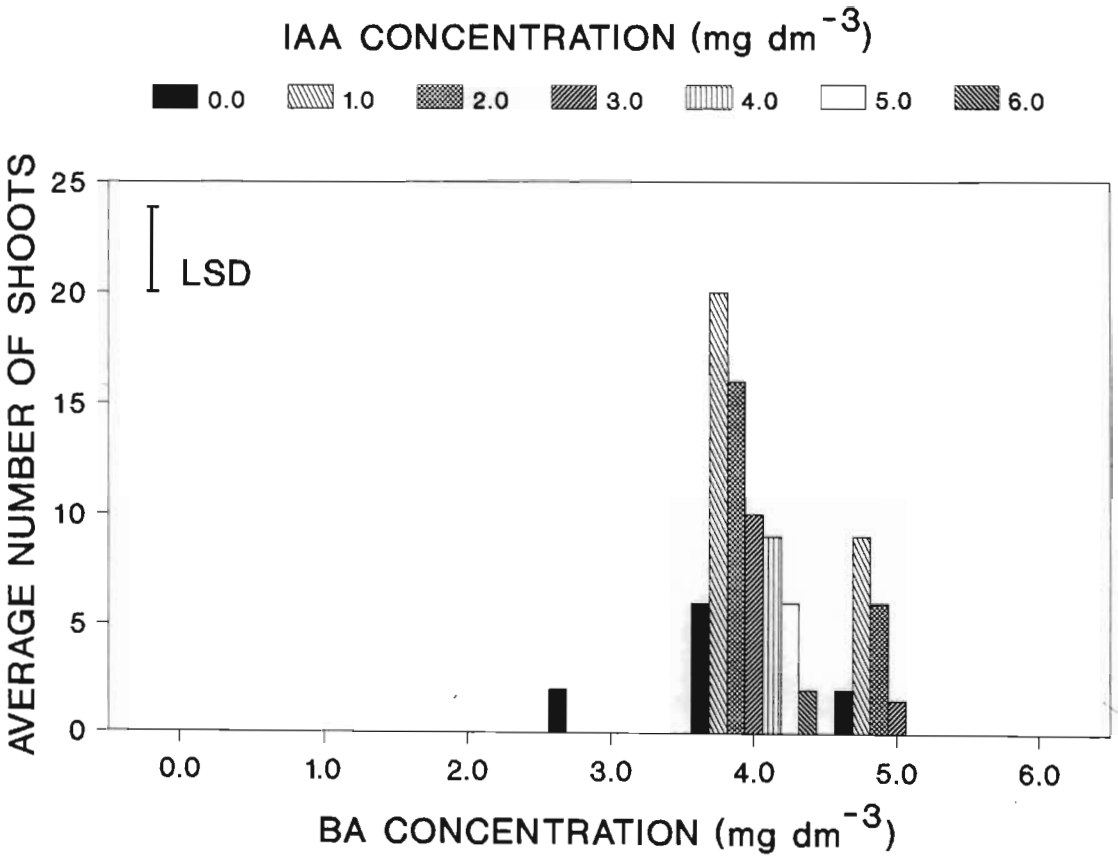


**FIGURE 3.6** : The average number of adventitious shoots formed per callus explant of *L. peruvianum* using 49 factorial combinations of BA and IAA.

### 3.3.2 The effect of zeatin and IAA on shoot formation

#### 3.3.2.1 Shoot induction on leaf tissue of *L. esculentum*

Leaf explants of *L. esculentum* regenerated shoots when cultured on a medium with 2.0 and 3.0 mg $dm^{-3}$ zeatin. At 2.0 mg $dm^{-3}$ zeatin, IAA brought about greatest shoot production at 1.0 mg $dm^{-3}$ (Figure 3.7). No significant differences were found between any other treatment with zeatin and IAA.

#### 3.3.2.2 Shoot induction on callus tissue of *L. esculentum*

No shoot regeneration occurred with any combination of zeatin and IAA in callus tissue of *L. esculentum*.

#### 3.3.2.3 Shoot induction on leaf tissue of *L. peruvianum*

In leaf tissue of *L. peruvianum* shoot regeneration was found at 2.0 and 3.0 mg $dm^{-3}$ zeatin (Figure 3.8). IAA concentration also had an effect on the regeneration of adventitious shoots since a maximum average number of

adventitious shoots were formed using IAA at 0.1 mg $dm^{-3}$. Concentrations of IAA higher than 0.1 mg $dm^{-3}$ caused a decline in shoot production and no shoots were induced at IAA concentrations of 3.0 mg $dm^{-3}$ and upwards. No significant differences were found between 0.1 and 1.0 mg $dm^{-3}$ IAA.

### 3.3.2.4 Shoot induction on callus tissue of *L. peruvianum*

In callus tissue of *L. peruvianum* shoot regeneration occurred only at 2.0 mg $dm^{-3}$ zeatin (Figure 3.9). IAA concentrations caused greatest shoot production at 0.1 mg $dm^{-3}$. No significant differences were found between 0.1 and 1.0 mg $dm^{-3}$ IAA.

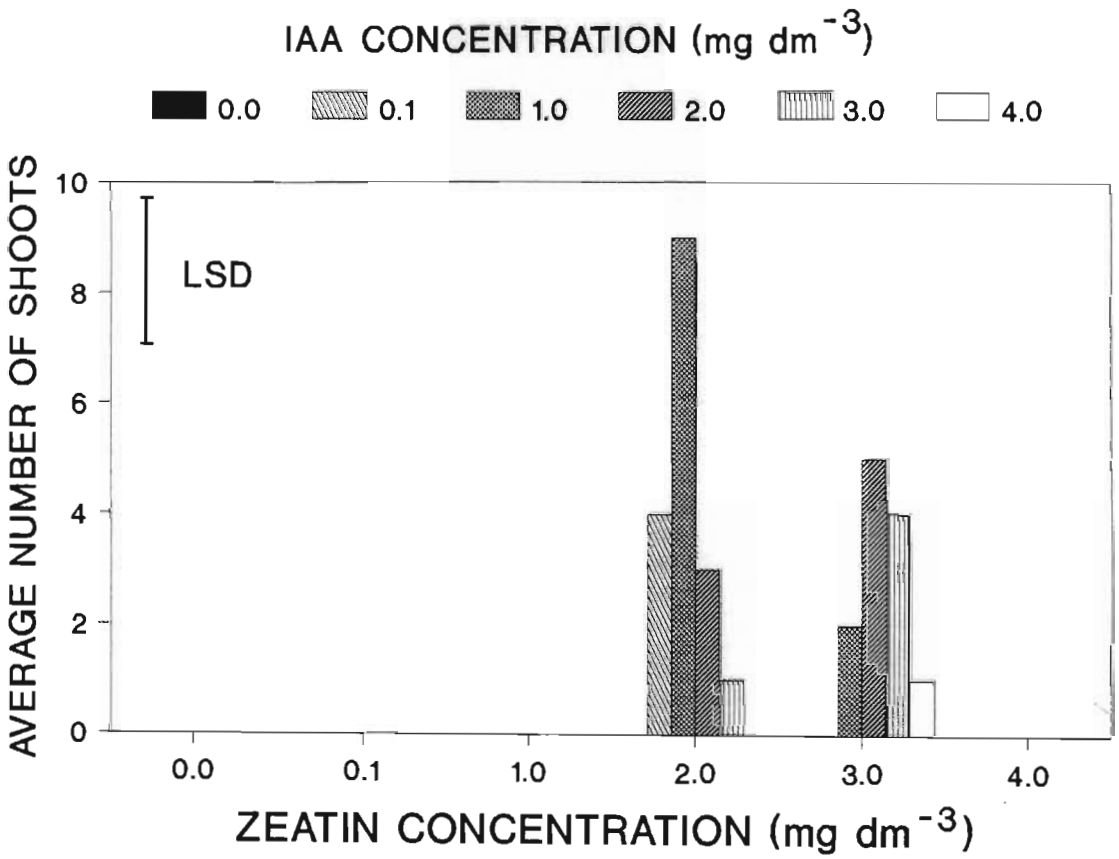


**FIGURE 3.7** : The average number of adventitious shoots formed per leaf explant of *L. esculentum* using various factorial combinations of zeatin and IAA.

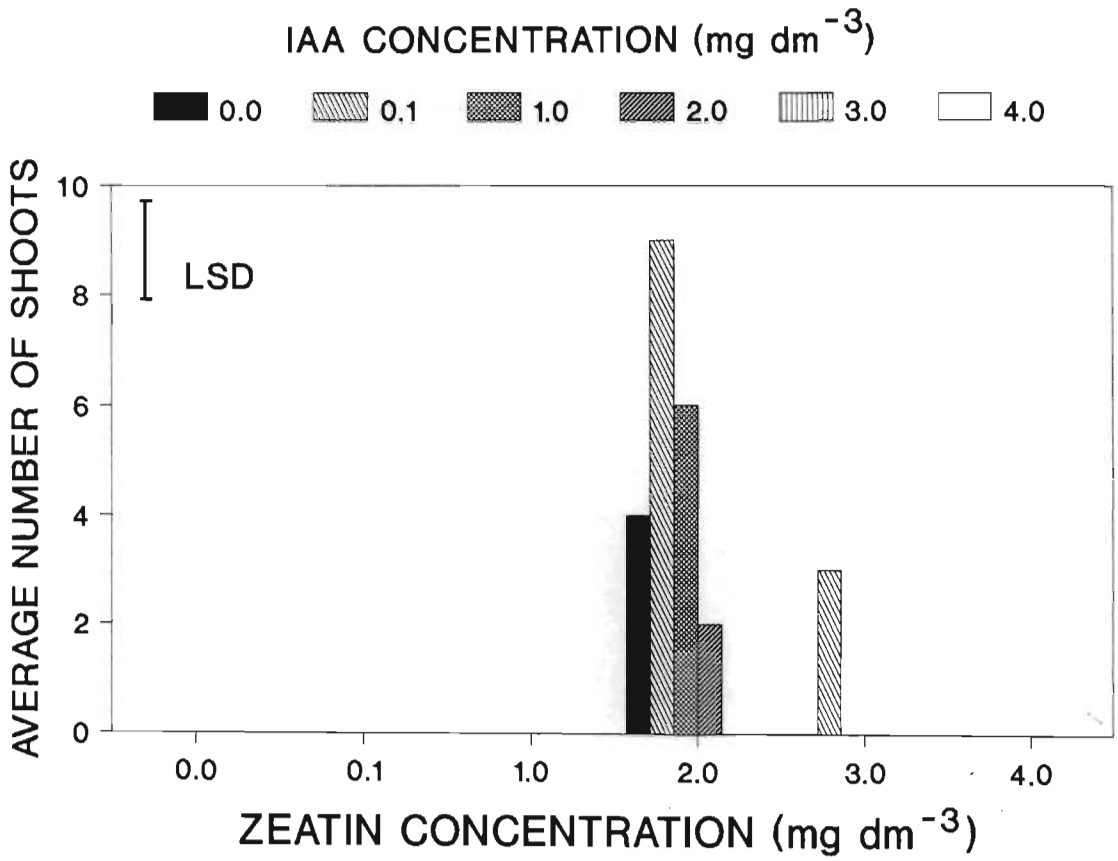


**FIGURE 3.8** : The average number of adventitious shoots formed per leaf explant of *L. peruvianum* using various factorial combinations of zeatin and IAA.

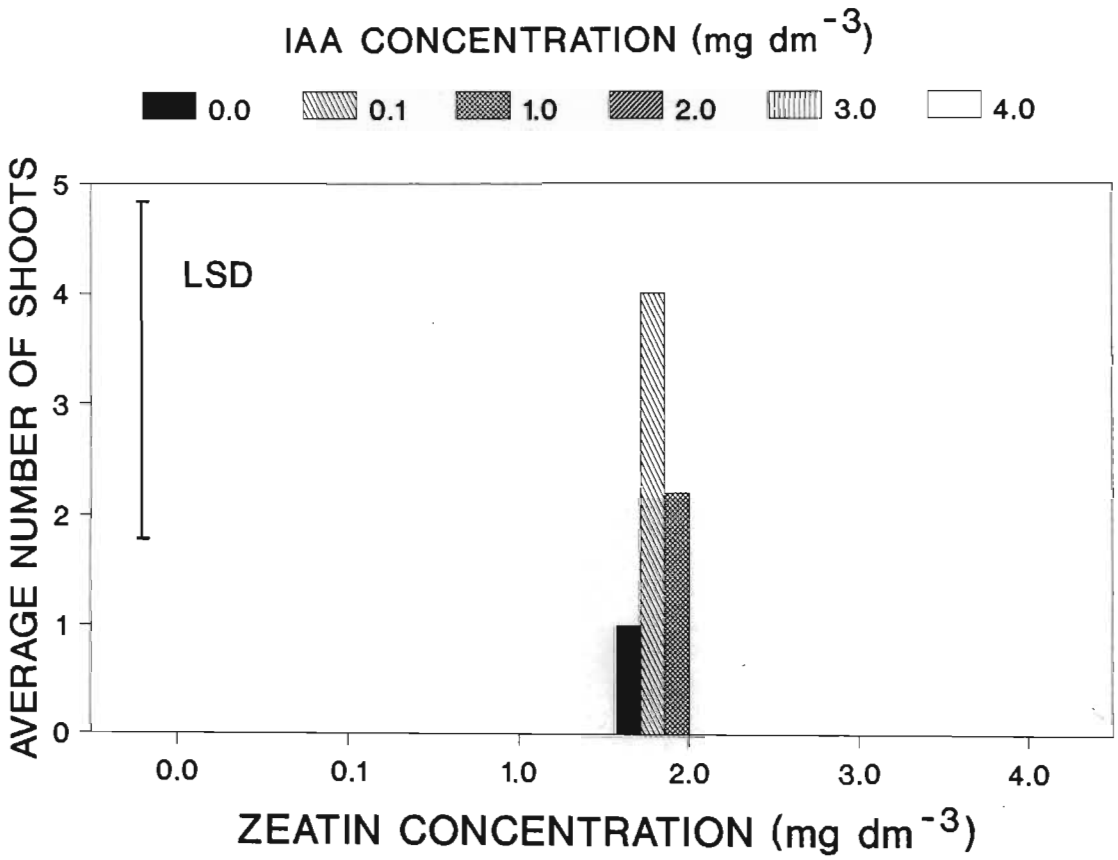


**FIGURE 3.9** : The average number of adventitious shoots formed per callus explant of *L. peruvianum* using various factorial combinations of zeatin and IAA.

## 3.4 DISCUSSION

Thorpe (1982) have shown that the balance between auxins and cytokinins is one of the major controlling factors in morphogenesis. These studies have also showed on the importance of the endogenous auxin:cytokinin balance. PALNI, BURCH and HORGAN (1988) have shown that cytokinin metabolism is affected by auxin concentration in the tobacco pith-explant system. They found that NAA caused an increased breakdown in zeatin riboside probably because of the regulatory effect it has on the enzyme "Cytokinin oxidase". The significant differences between the different media of the factorial experiments is therefore no surprise.

With leaf tissue of *L. esculentum* and *L. peruvianum* the stimulatory effect of IAA concentration on the regeneration of shoots, up to a certain level, can be ascribed to the disturbance of endogenous IAA levels by high exogenous IAA levels so much as to cause an imbalance in the cytokinin:auxin ratio (KARTHA, CHAMPOUX and PAHL, 1977). KARTHA, GAMBORG, SHYLUK and CONSTABEL (1976) also showed that the effect of BA on the production of shoots was IAA dependent up to a supra optimal level of IAA.

Similar results were found with increasing BA concentrations on leaf discs of *L.*

*esculentum* (BEHKI and LESLEY, 1976). Considering the above factors a BA:IAA ratio of 4:1 was found to be the best for shoot regeneration on leaf discs of *L. peruvianum*, whereas a 4:0.1 ratio of BA to IAA was found to be necessary for the production of adventitious shoots from callus tissue of *L. peruvianum*.

When comparing the morphogenetic response between *L. peruvianum* and *L. esculentum* it would appear that the former had the highest morphogenetic potential (TAL, DEHAN and HEIKIN, 1977). This was also found to be the case in this study.

Leaf callus of *L. peruvianum* required a low IAA concentration for shoot regeneration. BRANCA, BUCCI, DOMIANO, RICCI, TORELLI and BASSI (1991) found that auxins often obey the minumum requirement rules for activity but, that auxin structure plays a minor role in morphogenesis. It was also found *in vitro* that various explant types can be significantly different in their response to growth regulators (BEHKI and LESLEY, 1976), hence the differences in IAA requirements between callus and leaf tissue of *L. peruvianum*.

A total of 150 different media were tested for the regeneration of shoots from tissue of *L. esculentum* by VNUCHKOVA (1977). These studies indicated that a high

cytokinin:auxin ratio are necessary for regeneration in the cultivars tested. Allthough the response is specific to the genotype, similar responses were detected during this study and the growth regulator requirements for the cultivar Rodade also display a similar pattern. FRANKENBERGER, HASEGAWA and TICGHELAAR (1981) noted quantitative differences between the genotypes with respect to growth regulators.

COMPTON and VEILLEUX (1991) found that IAA was necessary for plant regeneration in tomato and that there is a specific IAA concentration above which a decline in shoot production was detected. This study also showed that IAA was necessary for shoot induction in Rodade. When IAA was increased above 4.0 mg $dm^{-3}$ a decline in the average number of shoots was noted. This phenomenon was also reported for the tomato cultivar Ohio 7814 (COMPTON and VEILLEUX, 1991). These results are contradictory to the findings of NOVAK and MASKOVA (1979) where IAA had little effect on the regeneration of tomato shoots.

By gradually increasing the BA concentration, an optimal point between 4.0 and 5.0 $mg.dm^{-3}$ was established with respect to shoot regeneration in cv. Rodade. This finding is in agreement with the work of NOVAK and MASKOVA (1979) as well as that of GUNAY and RAO (1980). The fact that shoot regeneration could be

obtained at IAA levels between 3.0 and 4.0 $mg.dm^{-3}$ showed that both BA:IAA ratios of 4:4 or 3:5 could be used for effective shoot regeneration. UDDIN, BERRY and BISGES (1988) also found that some cultivars produced shoots on several different media, while others were very specific with respect to their requirements for growth regulators.

No regeneration of shoots was found on callus of *L. esculentum*. Similar results were reported by HERMAN and HAAS (1978) with leaf callus of *L. esculentum*. These authors found that shoots and leaves failed to be produced from callus from which all visible differentiated tissue was removed.

Upon closer examination of the cytokinins used in these experiments it was found that BA was superior to zeatin with regard to the regeneration of adventitious shoots in the tissue types tested. GUNAY and RAO (1980) showed that IAA and BA were superior to IAA and kinetin. Zeatin was also shown to be able to induce shoots in the absence of an exogenously applied auxin in tomato cultures (MONTAGNO, LINEBERGER and BERRY, 1989). The opposite was true for leaf and callus tissue of *L. peruvianum* and leaf tissue of *L. esculentum* in that both a cytokinin (BA) as well as an auxin (IAA) were necessary to induce shoots.

This study have shown that it is possible to regenerate shoots from leaf discs and callus of *L. peruvianum* and leaf discs of *L. esculentum*. Optimum concentrations of IAA and BA, at which all future experiments will be performed for leaf and callus tissue, have been determined (Table 3.4). Since the optimal concentration for the regeneration of shoots from callus of *L. esculentum* is genotype dependent and different concentrations between 1.0 and 22.0 $mg.dm^{-3}$ have been used by different authors (TAL, DEHAN and HEIKIN, 1977; DHRUVA, RAMAKRISHHIVAN and VAIDYANATHAM, 1978; BEHKI and LESLEY, 1976; COMPTON and VEILLEUX, 1991) a BA:IAA ratio of 4:0.4 (10:1) has been selected for all subsequent experiments using callus of *L. esculentum* (Table 3.4).

**TABLE 3.4** : Growth regulator concentrations suggested for use in future research.

| Species | Tissue type | BA ($mg.dm^{-3}$) | IAA ($mg.dm^{-3}$) |
|---|---|---|---|
| *L. esculentum* | Leaf discs | 4.0 | 4.0 |
| | Callus | 4.0 | 0.4 |
| *L. peruvianum* | Leaf discs | 4.0 | 1.0 |
| | Callus | 4.0 | 0.1 |

# CHAPTER 4

# THE EFFECTS OF 6-BENZYLADENINE PULSES ON LEAF AND CALLUS TISSUE OF *L. ESCULENTUM* CV. RODADE AND *L. PERUVIANUM*.

## 4.1 INTRODUCTION

Although the effect of exogenous cytokinins on excised plant organs is well documented (LAMPUGNANI, FANTELLI, LONGO, LONGO and ROSSI, 1981) little is known about the absorption and the degree of exposure to BA that is necessary to induce the formation of adventitious shoots. Shoot formation is further complicated by the effects of attached differentiated tissue where induction of shoots is dependent on the presence of such tissue for at least the beginning of cultivation *in vitro* (SHARMA, BHOJWANI and THORPE, 1991). Although this factor is often described to be auxin-like, evidence exists for it to be correlated with the presence of cytokinins and the metabolism thereof (HERMAN and HAAS, 1978).

LAMPUGNANI, FANTELLI, LONGO, LONGO and ROSSI (1981) found a correlation between the levels of BA in watermelon cotyledons and the magnitude of the hormonal response, 4 days after BA-uptake. This relationship was true regardless of the time cultured in the presence of BA. Pulse treatments of relative short duration (2 h) with optimal BA concentrations followed by subculture on hormone free media produced a satisfactory yield of shoots in *Picea abies* (BORNMAN and VOGELMANN, 1984). GOLDFARB, HOWE, BAILEY, STRAUSS and ZAERR (1991) found relative short BA pulses and relative high BA concentrations to be necessary to produce adventitious buds in cotyledons of Douglas-fir. In contrast to these findings, NORDSTROM and ELIASSON (1986) found BA to be necessary as a continuous source for apple shoots to form *in vitro*.

Although the use of a solid medium is very popular, evidence also exists that a liquid medium can be superior to a solid medium with regard to shoot regeneration. Embryo production in anther cultures of cauliflower was improved by substitution of a solid medium with a liquid medium (YANG, CHAUVIN and HERVE, 1992). This effect was also observed by CHRAIBI, CASTELLE, LATCHE, ROUSTAN and FALLOT (1992) for mature cotyledons of sunflower.

Two methods of cytokinin application is therefore common. Explants may be placed on solid nutrient medium containing BA (GUNAY and RAO, 1980) for extended periods of time or alternatively, explants may be pulsed by incubation on solid or in liquid medium containing normal or high cytokinin concentrations (VOGELMANN, BORNMAN and NISSEN, 1984) and subsequently transferred to hormone free media.

In the following experiments the effects of BA pulses on shoot formation was studied in leaf and callus tissue of both *Lycopersicon esculentum* cv. Rodade and *Lycopersicon peruvianum*. As the optimal BA and IAA concentrations for the regeneration of adventitious shoots from all four tissue types were already determined previously (Chapter 3), experiments were conducted using optimal BA concentrations in solidified and liquid media. In addition optimal and higher than optimal BA concentrations were tested in liquid media. In these experiments the most effective pulse times as well as BA application methods were investigated in an attempt to determine the pulse length necessary to induce the production of adventitious shoots as well as the speed of induction. The results from the four tissue types were compared in an attempt to determine the reason for the recalcitrance of tomato callus to regenerate shoots *in vitro*.

## 4.2 MATERIALS AND METHODS

### 4.2.1 The production of leaf material

Leaf and callus tissue of both *L. esculentum* cv. Rodade and *L. peruvianum* were prepared as described earlier (Chapter 3).

### 4.2.2 The production of callus

Callus of both species were produced and maintained as described previously (Chapter 3).

### 4.2.3 Preparation of material for experiments

Four-week-old leaves were removed from sterile plantlets, the proximal and distal leaf ends were removed aseptically. In experiments where callus was used, green calli were cut into approximately 5x5x5 mm blocks and transferred to the appropriate experimental medium.

### 4.2.4 The effect of BA pulses over 40 days on solid and in liquid media on the induction of adventitious shoots on leaf and callus tissue

Leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were used in these experiments. In the experiment with solid medium, MSO medium supplemented with 4.0 mg $dm^{-3}$ BA and 4.0 mg $dm^{-3}$ IAA were used for leaf discs of *L. esculentum*, whereas MSO medium supplemented with 4.0 mg $dm^{-3}$ BA and 1.0 mg $dm^{-3}$ IAA were used for leaf discs of *L. peruvianum*. Callus of *L. esculentum* was inoculated onto a MSO medium supplemented with 4.0 mg $dm^{-3}$ BA and 0.4 mg $dm^{-3}$ IAA. As for callus of *L. peruvianum* a MSO medium supplemented with 4.0 mg $dm^{-3}$ BA and 0.1 mg $dm^{-3}$ IAA was prepared. The pH was adjusted to 5.8 with 1M KOH prior to autoclaving and media were dispensed in 10 $cm^3$ aliquots into specimen tubes (100 mm x 20 mm id.).

All four tissue types were transferred to the appropriate test medium under sterile conditions. Each day, for a period of 40 days, 20 explants of each tissue type were removed from the BA-containing medium and transferred to MSO medium without any growth regulators. All cultures were incubated as described earlier.

In the experiments with liquid media the media used for the different tissue types

had the same composition as described in the experiment with solid media, except that the agar was omitted. Media were dispensed as 100 $cm^3$ aliquots in 250 $cm^3$ Erlenmeyer flasks covered with foil and sealed with Parafilm (American Can Co.). A total of 40 flasks were prepared for each tissue type and the pH was adjusted to 5.8 with 1M KOH prior to autoclaving.

Each culture flask was inoculated with twenty explants and incubated on an orbital shaker at 80 r.p.m and a temperature of 25°C using the light regime described earlier. Each day, for a period of 40 days, 20 explants of each tissue type were removed from the BA-containing liquid medium and transferred to a solid MSO medium containing no growth regulators. All cultures were incubated as described earlier.

### 4.2.5 The effect of hourly BA pulses with supra optimal BA concentrations on the induction of adventitious shoots on leaf and callus tissue

Leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were used in this experiment. The four tissue types were exposed to different BA concentrations as well as different incubation times.

The BA exposure media consisted of 0, 10, 100 and 1000 μM BA disolved in $DDH_2O$ and sterilized in an autoclave for 20 minutes. Explants of all four tissue types were inoculated into one of these media on an orbital shaker and kept at 80 r.p.m at a temperature of 25°C under cool white fluorescent tubes with an intensity of 25 μmol $m^{-2}$ $s^{-1}$. Twenty explants were removed every hour, for a total of 6 hours, from the different BA-containing media. They were then washed in sterile $DDH_2O$ and placed on solid MSO without BA. All cultures were incubated as described earlier.

### 4.2.6 The effect of daily BA pulses with supra optimal BA concentrations in liquid media on the induction of adventitious shoots on leaf and callus tissue

Leaf and callus tissue of both *L. esculentum* and *L. peruvianum* were used in this experiment. The four tissue types were exposed to different BA concentrations as well as different incubation times.

The BA exposure media consisted of 0, 10, 100 and 1000 μM BA dissolved in a liquid MSO medium and sterilized in an autoclave for 20 minutes. Explants of all four tissue types were inoculated into one of these media on an orbital shaker and

kept at 80 r.p.m at a temperature of 25°C under a 16h/8h light/dark regime provided by cool white fluorescent tubes with an intensity of 25 μmol $m^{-2}$ $s^{-1}$. Twenty explants were removed every day, for a total of 40 days, from the different BA containing media. They were then washed in sterile $DDH_2O$ and placed on solid MSO without any growth regulators. All cultures were incubated under conditions as described earlier.

### 4.2.7 Data collection and analysis

After each BA pulse, the explants were transferred to an MSO medium without growth regulators. Explants were incubated on this medium for as many days as were required to complete a 40 day total incubation period (eg. Explants that were exposed to BA-containing medium for 10 days were incubated on MSO medium without any growth regulators for a further 30 days before data was collected). The shooting response of each treatment was calculated as the number of explants that produced shoots as a percentage of the total number of explants used per treatment. Twenty replicates were used for each treatment and each experiment was repeated four times. For each treatment the sum, mean, standard deviation and least significant difference (LSD) ($p<0.01$) were determined.

## 4.3 RESULTS

### 4.3.1 The effect of BA pulses over a period of 40 days on solid medium on the induction of adventitious shoots on leaf and callus tissue

#### 4.3.1.1 Leaf tissue of *L. esculentum*

With leaf discs of *L. esculentum* incubated on a MS medium with BA (4.0 mg $dm^{-3}$) and IAA (4.0 mg $dm^{-3}$) a regeneration response of 60% was obtained after 31 days (Figure 4.1). The first shoots were observed after a 7 day pulse with BA. A gradual increase in regeneration response was observed from day 7 over the next 25 days. The 60% regeneration response appears to be the optimum response as the percentage shoots did not increase with longer pulse times. In the control treatment where leaf tissue were not exposed to BA no shoot formation was recorded. Exposure of leaf tissue to BA-containing medium between 1 and 6 days did not induce the production of adventitious shoots *in vitro*.

#### 4.3.1.2 Callus tissue of *L. esculentum*

Callus tissue of *L. esculentum* showed no shoot formation after any pulse length

on BA-containing medium. Callus grew normally without losing vigour or the light green colour associated with it.

#### 4.3.1.3 Leaf tissue of *L. peruvianum*

An optimum shooting response was observed after an 18 day BA pulse (Figure 4.2). The shortest possible pulse length was 5 days with a shooting response of 10%. Between 5 and 18 days a rapid linear increase in the percentage of shoots that were produced were recorded. In this experiment all the leaf tissue formed shoots after having been exposed to BA containing medium for 18 days.

#### 4.3.1.4 Callus tissue of *L. peruvianum*

The lowest shooting response was recorded after a 10 day pulse with BA (Figure 4.3). Ten percent of all calli produced shoots at this treatment. A maximum response of 80% was recorded in the treatment where callus tissue was pulsed with BA for 35 days. The increase in shoot production was linear between 10 and 35 days of incubation on BA-containing medium.

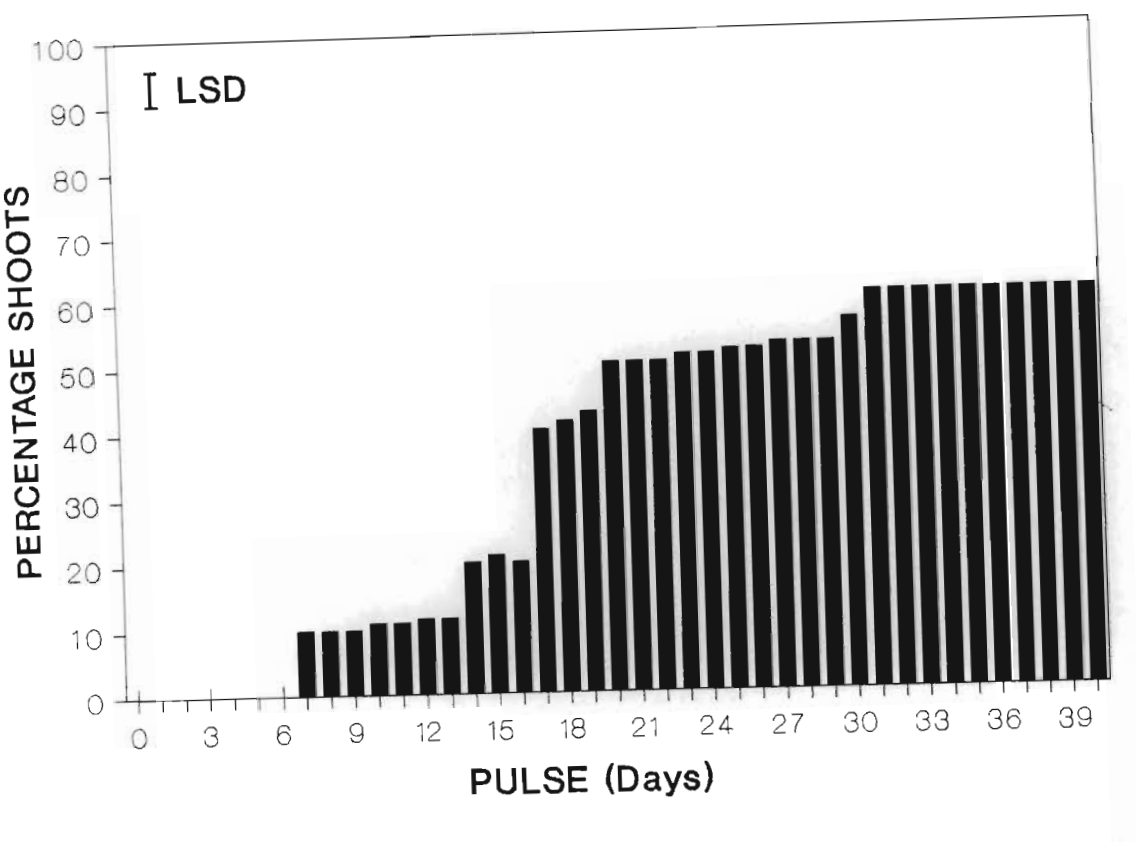


**FIGURE 4.1:** The effect of BA pulses on solid medium on shoot formation on leaf tissue of *L. esculentum* cv. Rodade.

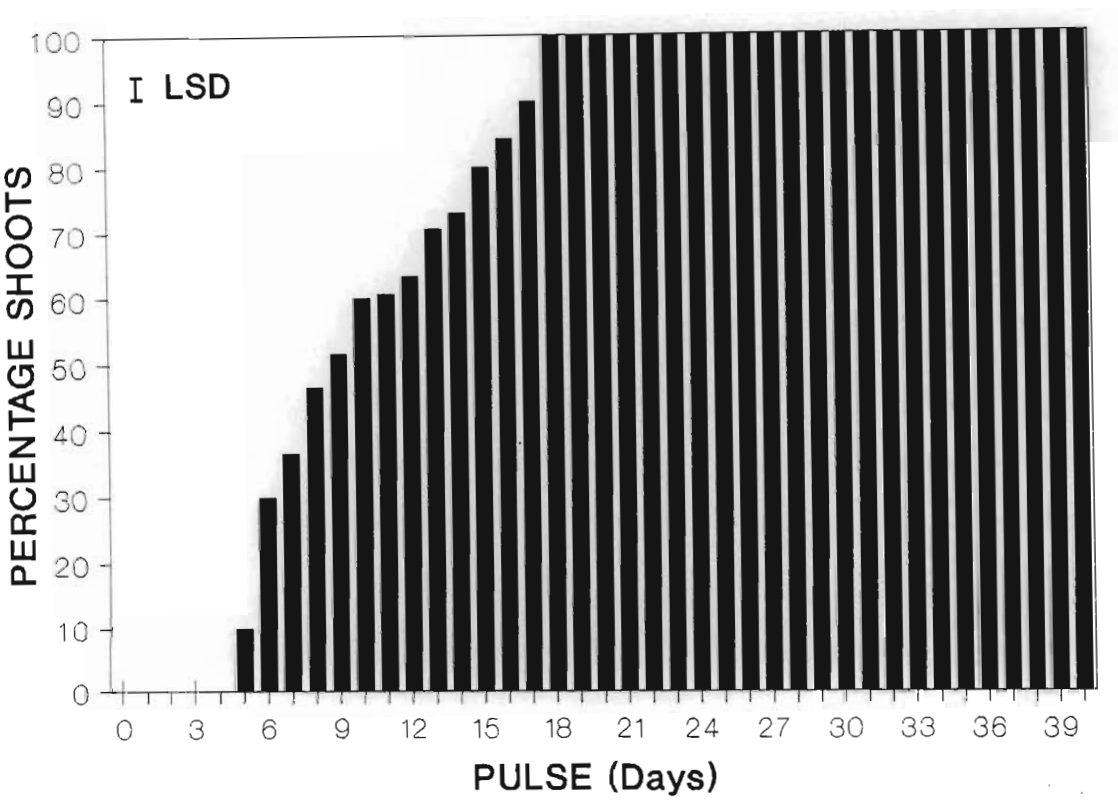


**FIGURE 4.2:** The effect of BA pulses on solid medium on shoot formation on leaf tissue of *L. peruvianum*.

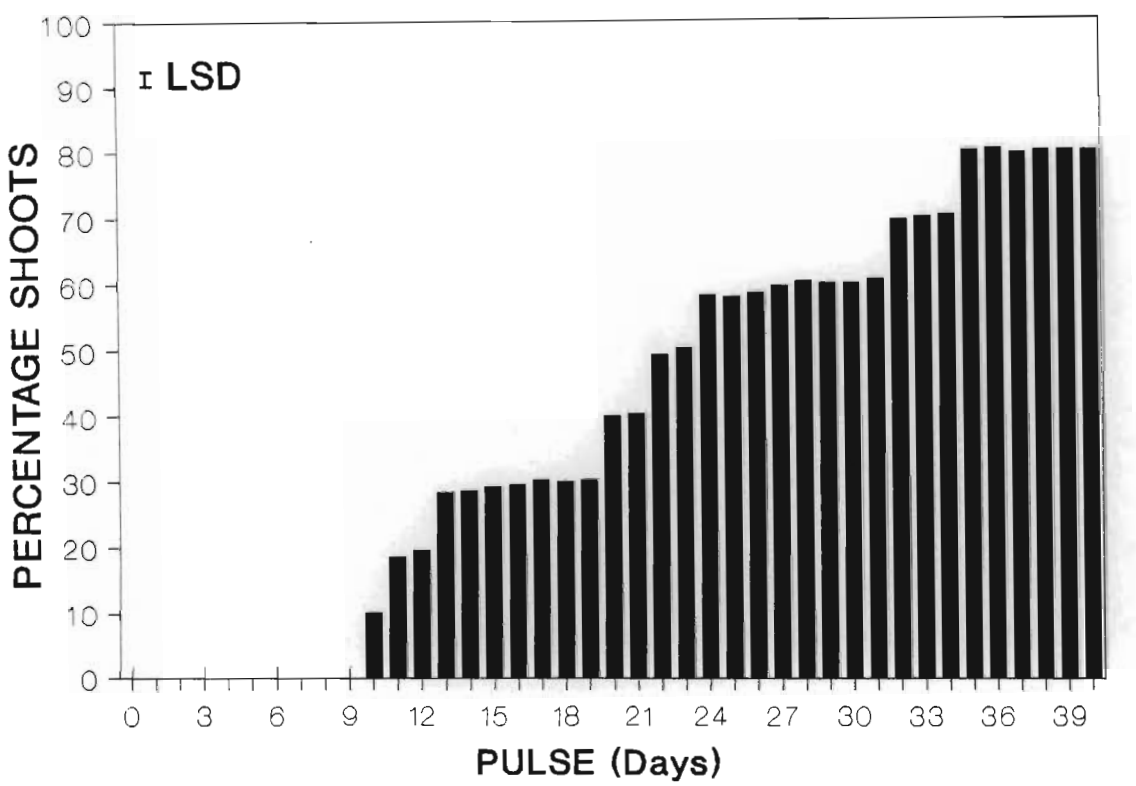


**FIGURE 4.3:** The effect of BA pulses on solid medium on shoot formation on callus tissue of *L. peruvianum*.

#### 4.3.1.5 Comparison of the different tissue types

A comparison of leaf and callus tissue of *L. peruvianum* (Table 4.1) showed that the maximum shooting response in leaf tissue was 20% higher than for callus tissue. The pulse time necessary to elicit a shooting response in leaf tissue of *L. peruvianum* was found to be less than for callus tissue. A five day difference was recorded between these two tissue types. Less time was required to elicit an optimum shooting response in leaf tissue of *L. peruvianum* than in callus tissue of this species resulting in a 17 day difference. A comparison of the time between the first induction of shoots and the pulse length necessary for the induction of the maximum number of shoots showed that leaf tissue regenerated shoots faster than callus tissue. These results showed that leaf tissue of *L. peruvianum* reacts faster to exposure to BA than callus tissue of the same species and that the maximum response is the highest in leaf tissue. The first shooting response of leaf and callus tissue were similar in *L. peruvianum*.

**TABLE 4.1:** Summary of data obtained from both leaf and callus tissue of *L. esculentum* and *L. peruvianum* on solid media.

| Reaction | *L. esculentum* | | *L. peruvianum* | |
|---|---|---|---|---|
| | Leaf | Callus | Leaf | Callus |
| Maximum shooting response (%) | 60 | -- | 100 | 80 |
| First shooting response observed (%) | 10 | -- | 10 | 10 |
| Shortest necessary pulse to obtain shoots (days) | 7 | -- | 5 | 10 |
| Shortest necessary pulse to obtain optimum results (days) | 31 | -- | 18 | 35 |
| Effective time of response (days) | 25 | -- | 14 | 26 |

No regeneration of shoots could be observed in callus of *L. esculentum.*

When comparing leaf tissue of *L. peruvianum* and leaf tissue of *L. esculentum* it was found that the highest shooting response was found in leaf tissue of *L. peruvianum* (Table 4.1). The shortest pulse necessary to induce adventitious shoots was two days longer for leaf tissue of *L. esculentum* than for the same tissue of *L. peruvianum*. The longest pulse with BA necessary to induce adventitious shoots

in leaf tissue of *L. esculentum* was 13 days longer than for leaf tissue of *L. peruvianum*. Upon comparing the time between the first occurrence of adventitious shoots and the longest necessary pulse time it was found that leaf tissue of *L. esculentum* took 11 days longer than leaf tissue of *L. peruvianum*. These results showed that leaf tissue of *L. esculentum* is slower to regenerate shoots than leaf tissue of *L. peruvianum*.

### 4.3.2 The effect of BA pulses over a period of 40 days in liquid medium on the induction of adventitious shoots on leaf and callus tissue

#### 4.3.2.1 Leaf tissue of *L. esculentum*

With leaf discs of *L. esculentum* incubated in a liquid medium an optimum shooting response of 70% was observed after a BA pulse of 35 days. The earliest shooting response was observed after a 7 day BA pulse. A gradual increase in percentage shoots was observed between pulse times from 7 to 35 days (Figure 4.4).

#### 4.3.2.2 Callus tissue of *L. esculentum*

Callus tissue of *L. esculentum* cv Rodade did not produce adventitious shoots over the experimental period.

#### 4.3.2.3 Leaf tissue of *L. peruvianum*

An optimum shooting response of 100% was recorded after a 20 day pulse with BA (Figure 4.5). A pulse time of 6 days was necessary to elicit the first response of 10%. Pulse lengths of between 6 and 20 days caused a rapid increase in shooting response.

#### 4.3.2.4 Callus tissue of *L. peruvianum*

The first shooting response of 10% was recorded after an 11 day pulse with BA (Figure 4.6). This increased to a maximum response of 80% after a 31 day pulse. Between days 11 and 31 a slow gradual increase in the shooting response was observed.

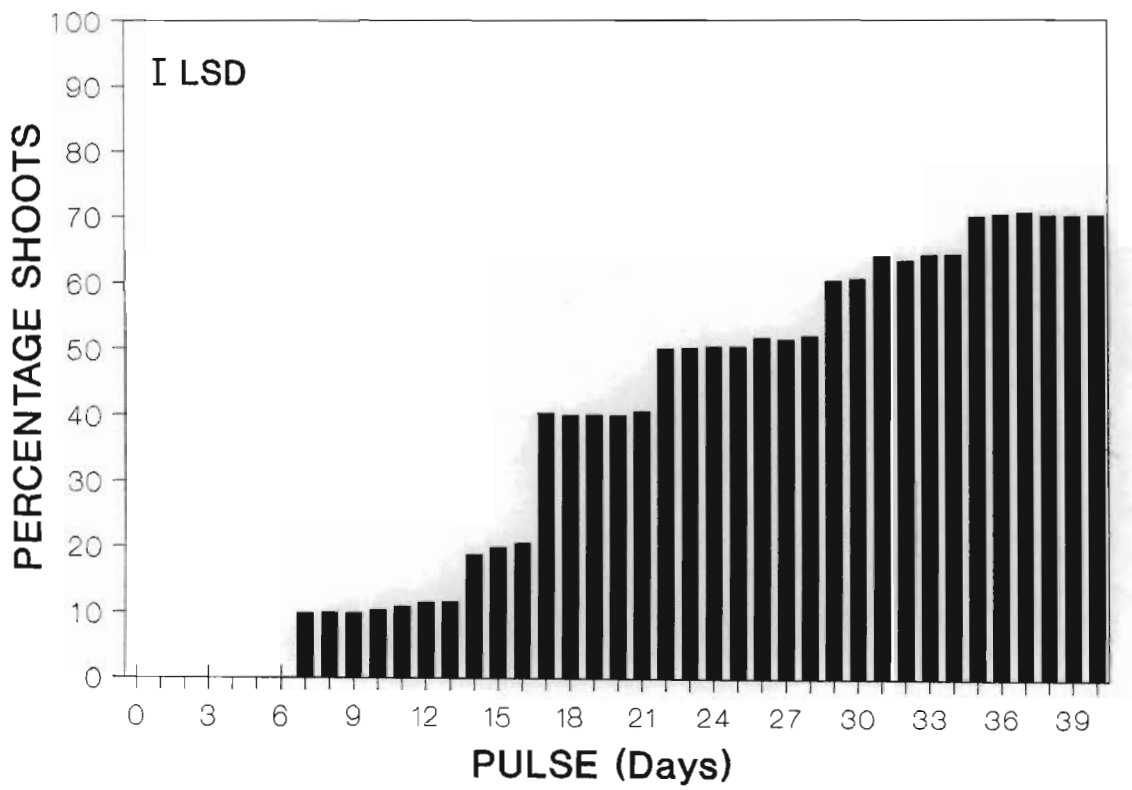


**FIGURE 4.4** : The effect of BA pulses in liquid medium on shoot formation on leaf tissue of *L. esculentum* cv. Rodade.

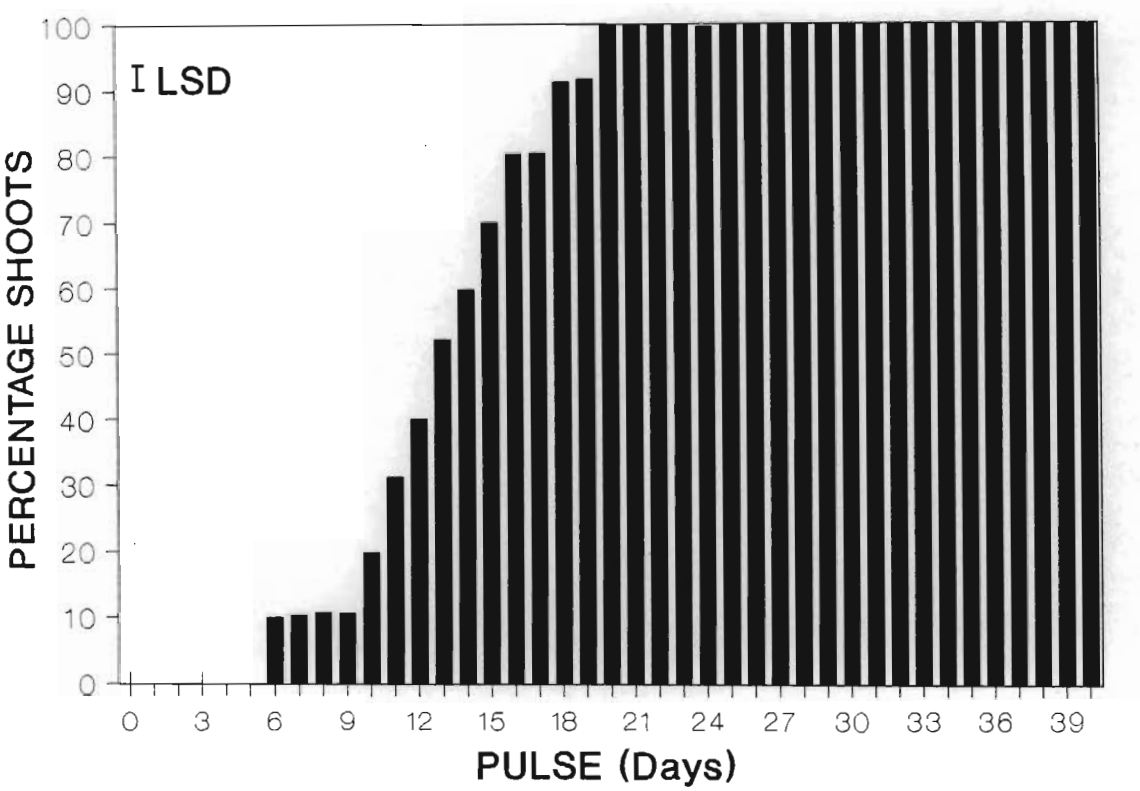


**FIGURE 4.5** : The effect of BA pulses in liquid medium on shoot formation on leaf tissue of *L. peruvianum*.

#### 4.3.2.5 Comparison of the different tissue types

A comparison of leaf and callus tissue of *L. peruvianum* (Table 4.2) showed that leaf tissue responded faster to BA pulses than callus tissue. The shortest pulse necessary to induce adventitious shoots was 6 days shorter for leaf tissue than for callus tissue of *L. peruvianum*. In order to induce an optimum percentage of adventitious shoots in *L. peruvianum* callus tissue had to be exposed for 11 more days to the BA containing medium than leaf tissue. When comparing the time between the first induction of shoots and the pulse time necessary for the induction of maximum shoots, it was also found that leaf tissue was the fastest responding tissue type. These results showed that leaf tissue of *L. peruvianum* reacts faster than callus tissue of *L. peruvianum* under the conditions tested.

In a comparison between leaf tissue of *L. esculentum* and leaf tissue of *L. peruvianum* a 30% higher production of adventitious shoots was found in *L. peruvianum*. The minimum pulse necessary to induce the production of adventitious shoots was two days longer for leaf tissue of *L. esculentum* than for *L. peruvianum*. To induce a maximum shooting response, leaf tissue of *L. esculentum* had to be pulsed with BA for 15 days longer than leaf tissue of *L. peruvianum*.

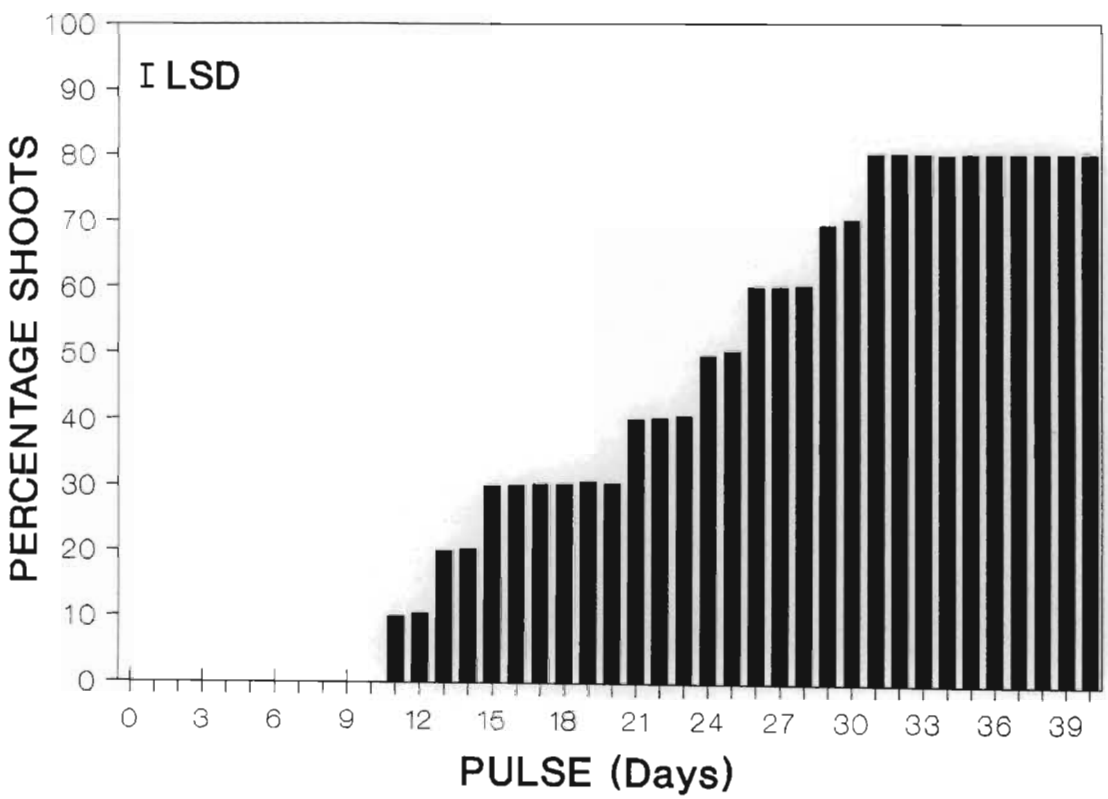


**FIGURE 4.6** : The effect of BA pulses in liquid medium on shoot formation on callus tissue of *L. peruvianum*.

**TABLE 4.2:** Summary of data obtained from both leaf and callus tissue of *L. esculentum* and *L. peruvianum* in liquid media.

| Reaction | *L. esculentum* | | *L. peruvianum* | |
|---|---|---|---|---|
| | Leaf | Callus | Leaf | Callus |
| Maximum shooting response (%) | 70 | -- | 100 | 80 |
| First shooting response observed (%) | 10 | -- | 10 | 10 |
| Shortest necessary pulse to obtain shoots (days) | 7 | -- | 5 | 10 |
| Shortest necessary pulse to obtain optimum results (days) | 35 | -- | 20 | 31 |
| Effective time of response (days) | 28 | -- | 14 | 20 |

The time between the first induction of adventitious shoots and the exposure necessary to induce the optimum production of shoots was twice as long for leaf tissue of *L. esculentum* than for leaf tissue of *L. peruvianum*. It was therefore also shown that leaf tissue of *L. esculentum* is slower in its response to BA than leaf tissue of *L. peruvianum*. Leaf tissue of both *L. esculentum* and *L. peruvianum* could be induced to produce adventitious shoots without having to be exposed to BA continuously. The first observed response was the same for both tissue types.

No regeneration could be observed in callus tissue of *L. esculentum*. However, these results show that less time is required in leaf tissue to elicit a response than in callus tissue and that a minimum pulse period could be found that produced maximum results. Leaf tissue of *L. esculentum* is slower in its response to BA than leaf tissue of *L. peruvianum* but the shortest necessary pulse with BA is less for leaf tissue of *L. esculentum* than for callus of *L. peruvianum*.

### 4.3.3 The effect of hourly BA pulses with supra optimal BA concentrations on the induction of adventitious shoots on leaf and callus tissue

#### 4.3.3.1 Leaf tissue of *L. esculentum*

Leaf tissue of *L. esculentum* showed no signs of adventitious shoot formation in any of the treatments applied. The leaf tissue remained healthy and green throughout the culture period but showed no signs of enlargement or the formation of callus.

#### 4.3.3.2 Callus tissue of *L. esculentum*

No adventitious shoots were observed in any treatment. The calli remained green and continued to enlarge throughout the culture period. At 100μM BA the calli at first turned brown and fresh dark green callus developed from the apparently dead callus after 10 days in culture.

#### 4.3.3.3 Leaf tissue of *L. peruvianum*

Leaf tissue of *L. peruvianum* produced adventitious shoots at 1000 μM BA in all the pulse treatments (Figure 4.7). After a one hour pulse with 1000 μM BA an optimum shooting response was observed. A gradual decrease in shooting was observed as the pulse treatment was increased to 6 hours.

#### 4.3.3.4 Callus tissue of *L. peruvianum*

Callus tissue of *L. peruvianum* showed no response to any pulse treatment. The callus remained green and continued to grow. Initially the callus turned brown to black after the initial BA treatment but from the apparently dead calli, green friable callus developed after 6 days on MSO without any growth regulators.

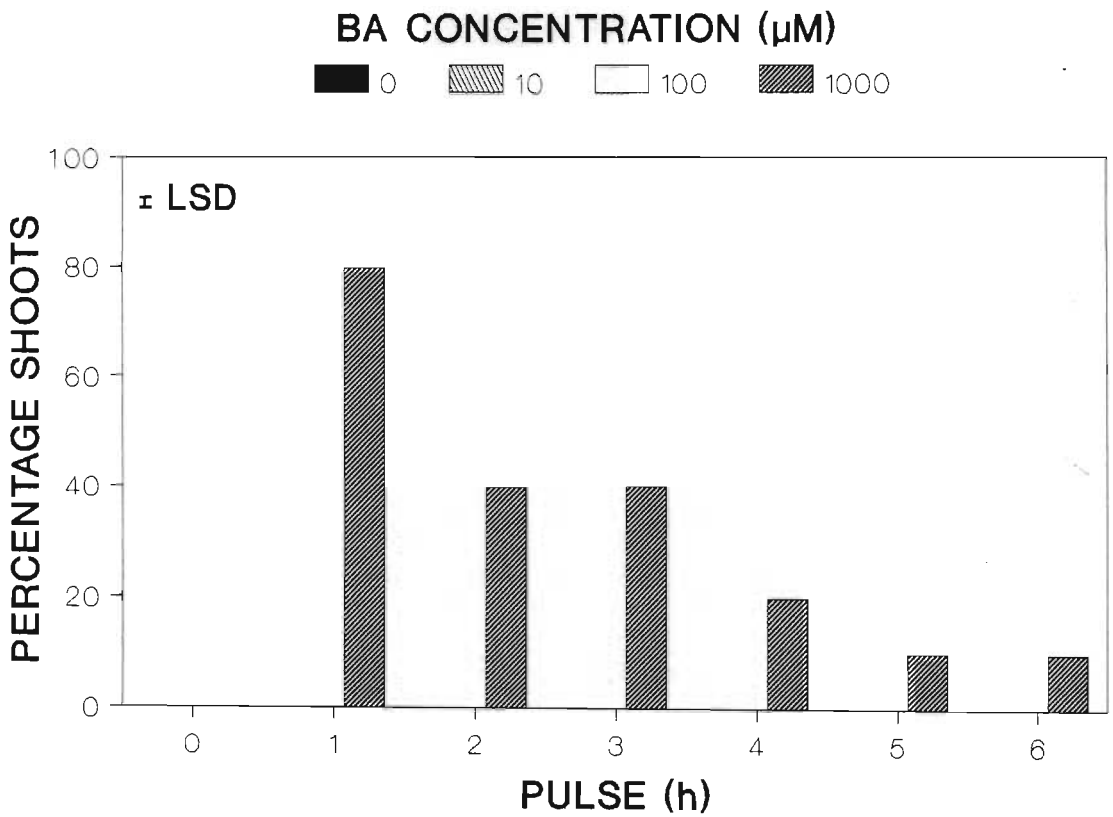


**FIGURE 4.7** : Shooting response of leaf tissue of *L. peruvianum* at different pulse times and concentrations of BA.

### 4.3.4 The effect of daily BA pulses with supra optimal BA concentrations in liquid media on the induction of adventitious shoots on leaf and callus tissue

Callus and leaf tissue of both *L. esculentum* cv. Rodade and *L. peruvianum* showed no signs of regeneration at any concentration or after any pulse length with BA during this experiment.

## 4.4 DISCUSSION

It was necessary to study the effect of pulse treatments with BA with regard to the morphogenetic response in tomato in order to determine the optimal conditions for regeneration. On solid media the response of tomato callus tissue differed greatly from that of tomato leaf tissue. Although slight callus production was noted on leaf tissue prior to regeneration, no regeneration was found in callus tissue consisting only of undifferentiated cells. This phenomenon was previously described for tomatoes by HERMAN and HAAS (1978) who reported that callus without any visibly attached differentiated tissue was unable to regenerate adventitious shoots. This study confirmed that it was not possible to regenerate adventitious shoots from undifferentiated callus of *L. esculentum* cv Rodade under the present experimental

conditions.

Unlike experiments with apple tissue (NORDSTROM and ELIASSON, 1986) a continuous exposure of leaf tissue of *L. esculentum* to BA was unnecessary for the production of adventitious shoots. The shortest possible pulse time with BA sufficient for the induction of shoots was found to be 7 days when at least 10% of all leaf explants regenerated shoots. A maximum response was obtained after a 31 day pulse with BA. These results showed that continuous subjection to BA is unnecessary for the induction of adventitious shoots. Pulse lengths of between 7 and 31 day caused a stepwise increase in the percentage shoots regenerated, indicating a period within which exposure to BA is beneficial to the induction of adventitious shoots. Similar results have been reported previously by DE VILLIERS, JANSE VAN VUUREN, FERREIRA and VAN STADEN (1993).

The method of cytokinin application also produced minor improvements in the regeneration response. Unlike results found with mature cotyledons of *Helianthus annuus* where liquid media proved to be better than a solid medium in terms of regeneration (CHRAIBI, CASTELLE, LATCHE, ROUSTAN and FALLOT, 1992), only a 10% improvement in maximum shooting response could be obtained in leaf tissue of *L. esculentum* subjected to BA pulses in liquid media when compared to

the same response on solid medium. To obtain a maximum response, the leaves had to be subjected to longer BA pulses in liquid media than on a solid medium. Callus tissue of *L. peruvianum* however, responded faster to liquid media than to solid media indicating that BA is either available to tissue for longer periods in liquid media than in solid media or that the uptake of BA may be improved by the liquid medium in this tissue type. This phenomenon was also described by YANG, CHAUVIN and HERVE (1992) where anther cultures of cauliflower showed improved regeneration in liquid culture.

In *Picea abies* cytokinin (BA) pulse treatments proved to be effective for the induction of adventitious shoots (BORNMAN and VOGELMANN, 1986). In leaf and callus tissue of *L. esculentum* no shoots were induced after BA pulse treatments with various concentrations of BA at different pulse lengths. This study indicated that when increasing the pulse time to days no shoots could be induced on any tissue type. Leaf tissue was very slow in its reaction to BA.

The wild tomato, *L. peruvianum*, was more responsive than *L. esculentum*. *L. peruvianum* is known to perform much better *in vitro* than *L. esculentum* with regard to regeneration (TAL, DEHAN and HEIKIN, 1977). In this study it was shown that a 100% regeneration of adventitious shoots could be obtained from leaf

tissue of *L. peruvianum*. As much as 80% of all callus explants produced adventitious shoots after pulse treatments with BA for 31 days indicating that even in undifferentiated cultures of *L. peruvianum* good regeneration ability exists. Although NOVER, KRANZ and SCHARF (1982) found a low morphogenetic potential in both *L. esculentum* and *L. peruvianum* the present study has shown that when subjected to the correct conditions, both tissue types of *L. peruvianum* is superior to any tissue type of *L. esculentum* cv. Rodade with respect to regeneration.

Both leaf and callus tissue of *L. peruvianum* were able to regenerate adventitious shoots on solid media. In leaf tissue of *L. peruvianum* the minimum pulse with BA required for the induction of adventitious shoots was two days less than for leaf tissue of *L. esculentum*, showing a faster response in *L. peruvianum* and also possibly a faster rate of BA uptake and metabolism. Continuous exposure to BA in order to optimize shoot production was therefore unnecessary. A stepwise increase in shoot induction with respect to BA pulse length indicates an exposure range where the amount of induction may be controlled by the pulse length (DE VILLIERS, JANSE VAN VUUREN, FERREIRA and VAN STADEN, 1993).

The method of cytokinin application did not alter the regeneration response

remarkably. Liquid media produced similar results than solidified media under identical conditions. Leaf tissue of *L. peruvianum* was the only tissue type that regenerated shoots after the application of short cytokinin pulses. As with cotyledons of Douglas-fir (GOLDFARB, HOWE, BAILEY, STRAUSS and ZAERR, 1991) relatively high cytokinin (BA) concentrations were required to induce the production of adventitious shoots. This possibly occurred because of the faster response to BA pulses that was observed in tissue cultures of *L. peruvianum*.

# CHAPTER 5

# THE UPTAKE AND METABOLISM OF 6-BENZYLADENINE BY LEAF AND CALLUS TISSUE OF *L. ESCULENTUM* CV. RODADE AND *L. PERUVIANUM*

## 5.1 INTRODUCTION

Since the classic research of Skoog and Miller with tobacco pith cultures, it is known that *in vitro* organogenesis of many plant species is causally dependent on the exogenous cytokinin/auxin balance in the culture medium (SKOOG and MILLER, 1957). This situation is apparently also true for endogenous levels of plant growth regulators as increased levels of endogenous cytokinins are positively correlated with increased shoot production (BURCH and STUCHBURY, 1987). Working with $^{14}C$-marked cytokinins a correlation was found between the level of radioactivity present in the plant tissue and the magnitude of the hormonal response that is observed after uptake of the labelled cytokinin (LAMPUGNANI, FANTELLI, LONGO, LONGO and ROSSI, 1981). Few workers have examined the long-term uptake of BA in leaf and callus tissue. NORDSTROM and

ELLIASSON (1986) reported a linear uptake of BA in apple shoot cultures. BIONDI, CANCIANI and BAGNI (1984) found a rapid uptake of BA over the first part of a 25 day incubation period whereafter a decrease in uptake was recorded in elm shoots. This decrease was ascribed to less BA being available in the medium. It was also shown by BLAKESLEY and CONSTANTINE (1992) that different species have a different rate of BA uptake and metabolism.

VAN STADEN and MALLETT (1988) found that rootless shoots and roots of intact tomato plants metabolized BA rapidly and that differential metabolism exists within the different tissue types. BAYLEY, VAN STADEN, MALLETT and DREWES (1989) using various tomato organs indicated that roots contained the most extensive range of metabolites. These compounds included an unknown metabolite, adenine as well as the riboside, nucleotide and 9-glucoside of BA. The 9-glucoside was found only in the roots, whereas the stem tissue yielded benzyladenosine-5'-monophosphate and benzyladenosine. Leaf tissue yielded the unknown metabolite and adenine. It was concluded that various organs of the tomato metabolized BA differently. NANDI, PALNI, LETHAM and WONG (1989) reported the natural occurrence of BA its riboside and corresponding nucleotide in primary crown gall tumours of tomato. Differences in the metabolism of BA was also detected between different species. BLAKESLEY and CONSTANTINE

(1992) investigated the metabolism of BA in shoot cultures of a range of species and found that the pattern of BA metabolism varied considerably between species as did the level of metabolites formed. These results clearly show that under identical *in vitro* conditions a marked variation in the uptake and metabolism of BA occurs between different plant organs or tissues from a plant, or from different plant species.

BLAKESLEY, LENTON and HORGAN (1991) reported that in shoot cultures of *Gerbera jamesonii* [3G]BA, [9G]BA, [9R]BA and a novel compound were produced as metabolites of BA. The 9-riboside of BA was the principal metabolite in petioles, apices and callus. After longer periods of exposure the major metabolite found in callus was the 9-ribosylglucoside of BA whereas both the 3-glucoside and 9-glucoside accumulated in petioles and apices. Only the 3-glucoside of BA could be detected in the leaf tissue. These results suggests that specific metabolites of BA could be expected to accumulate in certain tissue types. In studies on the uptake and metabolism of BA by shoot cultures of *Musa* and *Rhododendron* it was reported that [9G]BA was the principal metabolite produced in both species (BLAKESLEY, 1991).

In an investigation of BA metabolism by decapitated roots of tomato it was found

that BA taken up by the roots was both ribosylated and glucosylated quite rapidly and that no unmetabolised BA could be detected in the roots after 24 hours (FORSYTH and VAN STADEN, 1987). It was concluded that a possibility exists for a correlation between the uptake and metabolism of BA and its efficiency *in vitro*. AUER, LALOUE, COHEN and COOKE (1992) characterised BA uptake and metabolism during the key stages of shoot organogenesis in *Petunia* leaf explants and emphasized the possibility that BA-ribotides might serve as a short term storage form prior to conversion to an active form. Alternatively, the ribotides themselves may constitute the active form of cytokinin. Thus it is clear that there is no indication to the precise function of the various metabolites.

It is evident that BA and its metabolites play key roles during organogenesis and that different organs and tissue types vary in their ability to take up and metabolize BA. In order to get a better understanding of the role of applied BA and to permit a more rational approach to the use of BA in the production of adventitious shoots *in vitro*, the uptake and metabolism of $^{14}$C-BA was studied in both leaf and callus tissue of the two tomato species *L. esculentum* cv. Rodade and *L. peruvianum*.

## 5.2 MATERIALS AND METHODS

### 5.2.1 The production of leaf material

Leaf material of both species were produced and cultured as described earlier (Chapter 3).

### 5.2.2 The production of callus

Callus tissue of both species were produced and cultured as described earlier (Chapter 3).

### 5.2.3 Preparation of material for experiments

Six-week-old leaves were removed from sterile plantlets, the proximal and distal ends of the leaves were cut away under aseptic conditions. In experiments where callus were used, green calli was cut into approximately 5 x 5 x 5 mm blocks and transferred to the appropriate medium.

### 5.2.4 Preparation of medium

In this study 8-$^{14}$C-benzyladenine with a specific activity of 1.85 MBq was added to an MSO medium to give an approximate activity of 81.5 kBq in each 10 $cm^3$ aliquot of medium. Unlabelled BA and IAA were also added to the medium to ensure the appropriate BA concentrations necessary for the growth of the different tissue types (Table 3.4).

### 5.2.5 Incubation of explants

The four different tissue types (leaf and callus tissue of *L. esculentum* and *L. peruvianum*) were inoculated onto the appropriate media containing BA for 1, 5, 10, 15, 20, 25, 30, 35 and 40 days respectively. All cultures were incubated under conditions as described earlier. At the end of each incubation time the material was removed from the medium and extracted for cytokinins.

### 5.2.6 Extraction of cytokinins

Each tissue sample was weighed and frozen immediately whereafter the plant material was homogenized and extracted overnight in 50 $cm^3$ 80% (v/v) ethanol.

The extract was then filtered through a 0.45 μm Millipore filter and concentrated to near dryness *in vacuo* at 35°C. The residue was resuspended in 500 $\mu dm^3$ 80% (v/v) methanol and filtered through a 0.2 μm Dynagard filter and again concentrated to dryness *in vacuo* at 35°C. The residues were then resuspended in 80% (v/v) HPLC grade methanol on the basis of 100 $\mu dm^3\ g^{-1}$ fresh mass extracted.

### 5.2.7 Determination of $^{14}$C-BA uptake

To determine the remaining radioactivity in the medium, the medium was warmed to 95°C and 0.5 $cm^3$ of the medium was added to 1 $cm^3$ of 30% (v/v) $H_2O_2$ containing 1% (v/v) ammonium hydroxide. This mixture was allowed to stand for overnight at 25°C to allow the agar to be dissolved. To destroy the remainder of the $H_2O_2$ the vials were heated to 60°C for 1 h. This mixture was then added to 10 $cm^3$ Ready Solv (Beckman) solution and the samples were counted in a Rackbeta 1219 liquid scintillation counter with correction for background.

To determine the radioactivity in the plant material, the plant material was carefully weighed and frozen immediately in liquid $N_2$. The tissue was then homogenized in 80% (v/v) ethanol. The extracts were filtered through filterpaper (Whatman no. 1) and the filtrate taken to near dryness *in vacuo* at 32°C. The extract was then re-

suspended in 1.5 $cm^3$ Beckman Ready-Solv scintillation fluid and the samples were counted in a Rackbeta 1219 liquid scintillation counter with correction for background.

Twenty samples of each treatment were analysed for all tissue types tested. The sum, mean and standard deviation were calculated and the least significant difference (LSD) ($p<0.01$) determined.

### 5.2.8 HPLC analysis of extracts

Aliquots of 25 $\mu dm^3$ of each extract (prepared as described above) were injected onto a Hypersil 5 ODS column (5 $\mu$m, $C_{18}$-bonded, 250 x 4 mm) and separated at a flow rate of 1 $cm^3\ min^{-1}$ using a Beckman System Gold HPLC system. Separation was achieved using an aqueous buffer of 0.2 M acetic acid, adjusted to pH 3.5 with triethylamine (LEE, MOK, MOK, GRIFFIN and SHAW, 1985). Samples were eluted with a linear gradient of methanol from 5 - 50% over 90 minutes. Fractions of 1 $cm^3$ were collected and used for the detection of radioactivity.

### 5.2.9 Determination of radioactivity

Each 1 $cm^3$ fraction recovered by HPLC was scanned for radioactivity. A 200 $\mu dm^3$ aliquot was taken from each 1 $cm^3$ sample collected and after the addition of 3 $cm^3$ Beckman EP Ready-Solv scintillant, the radioactivity was recorded on a Rackbeta 1219 liquid scintillation counter. A total of four separate analysis were performed for each tissue type.

### 5.2.10 TLC analysis of compounds

HPLC fractions associated with the retention time of BA, [9R]BA and [9R-MP]BA were each concentrated to near dryness *in vacuo* and re-dissolved in 200 $\mu$ $dm^{-3}$ methanol. The fractions were then loaded onto TLC plates (Merck, Silica gel, $PF_{254}$) and separated using n-butanol:25% ammonium hydroxide:water (BAW; 6:1:2; upper phase). Authentic BA, [9R]BA and [9R-MP]BA were used as standards.

## 5.3 RESULTS

### 5.3.1 Uptake and metabolism of $^{14}$C-BA by leaf tissue of *L. esculentum*

A rapid uptake of $^{14}$C-BA was recorded within the first 20 days of incubation on medium containing $^{14}$C-BA (Figure 5.1). A total of 87% of the available $^{14}$C-BA was detected in the tissue after 20 days. The uptake of $^{14}$C-BA slowed down between 21 and 40 days of incubation. In this period a further 9% of the available BA was absorbed.

A total of 6 HPLC-derived radioactive peaks were recovered from leaf tissue of *L. esculentum*. The radioactive fractions co-eluted with Ade in peak 1, [3G]BA in peak 2, [9R-MP]BA in peak 3, [9G]BA in peak 4, BA in peak 5 and [9R]BA in peak 6 (Table 5.1). Peak 3 also co-chromatographed with authentic [9R-MP]BA during TLC, peak 5 with authentic BA and Peak 6 with [9R]BA. In leaf tissue of *L. esculentum* only small amounts of radioactivity were associated with [9R]BA and [9R-MP]BA after one day of exposure to labelled BA (Figure 5.2).

After one day the most radioactivity co-eluted with authentic BA. Radioactivity was found to be associated with the retention times of [3G]BA, [9R-MP]BA,

[9G]BA, BA and [9R]BA after 5 days of exposure to $^{14}$C-BA. All six compounds were detected from day 10 to day 40 (Figure 5.3). A compound that had the same retention time as Ade was the last new radioactive peak detected during the course of the experiment. The amount of radioactivity associated with the retention time of BA decreased during the experiment. These results showed that metabolism of BA was slow during the first 24 h of exposure to $^{14}$C-BA. Small peaks of radioactivity associated with [9R-MP]BA and [9R]BA were found initially after incubation with $^{14}$C-BA. Compounds associated with the 3- and 9 glucoside of BA were formed later. The radioactive peak associated with [3G]BA reached an optimum after 25 days of exposure. Less radioactivity was detected at this retention time after longer exposures to $^{14}$C-BA. Radioactivity associated with peak 3 increased up to 15 days of exposure to $^{14}$C-BA. A decline in the amount of radioactivity associated with [9R]BA was found after 1 day of exposure to $^{14}$C-BA.

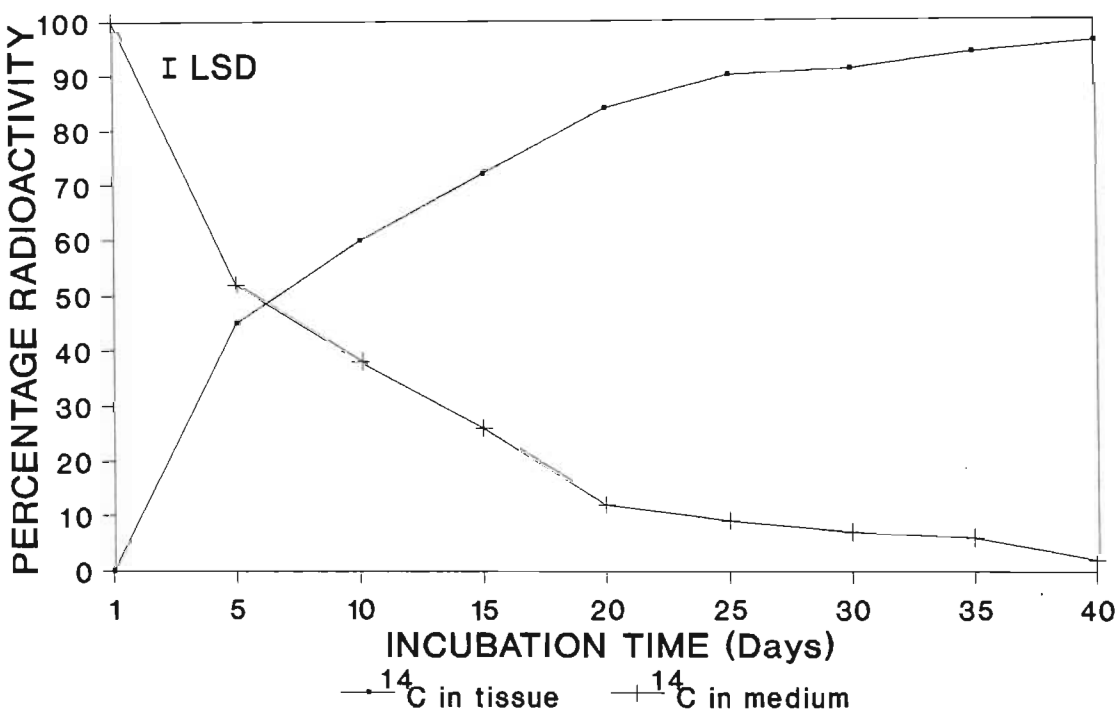


**FIGURE 5.1** : The uptake of $^{14}$C-BA over 40 days by leaf tissue of *L. esculentum* cv. Rodade.

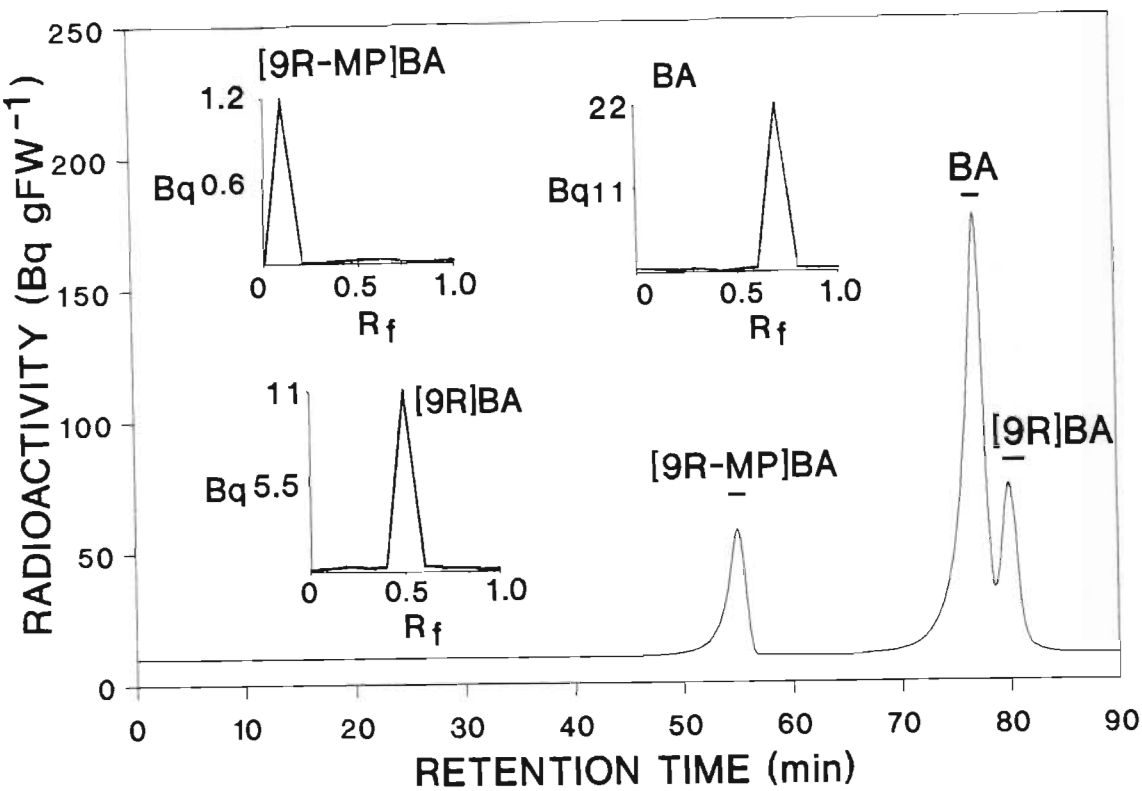


**FIGURE 5.2** : Radioactivity detected in an extract of leaf tissue of *L. esculentum* cv. Rodade after 1 day of incubation on a medium containing $^{14}$C-BA. The retention time of authentic standards are indicated. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

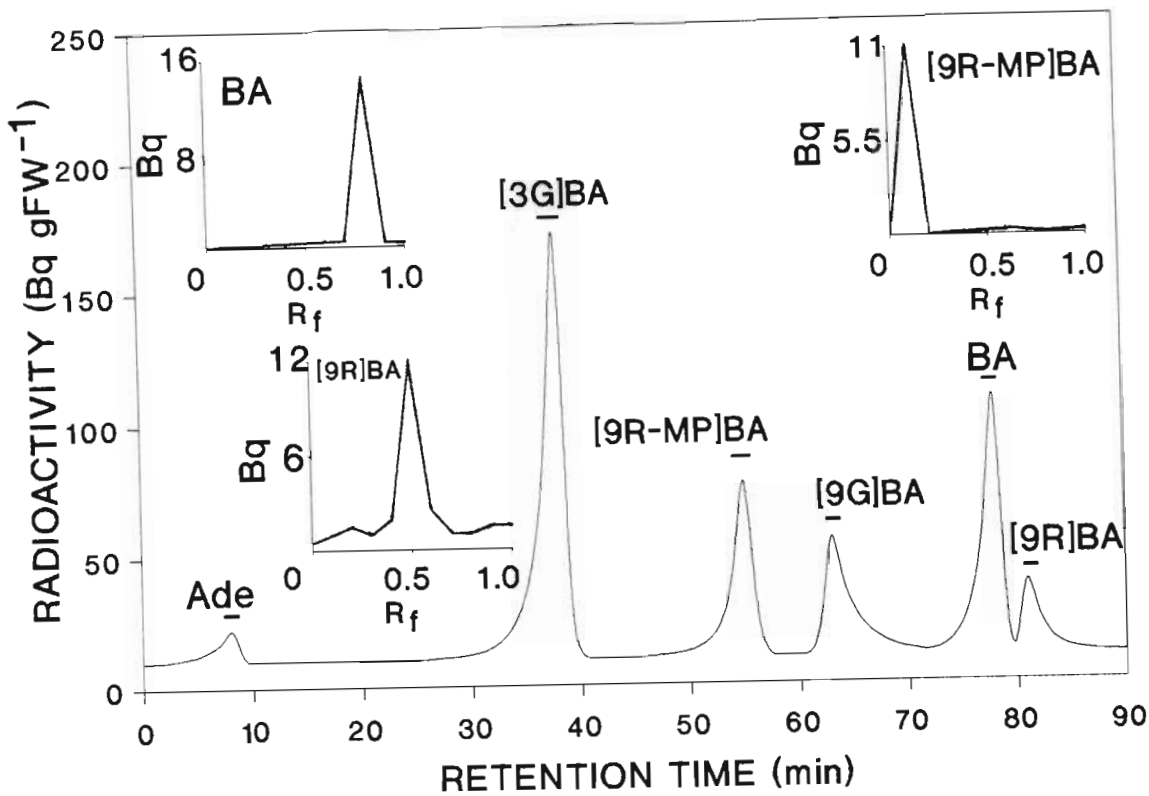


**FIGURE 5.3** : Radioactivity detected in an extract of leaf tissue of *L. esculentum* cv. Rodade after 10 days of incubation on a medium containing $^{14}$C-BA. The retention time of authentic standards are indicated. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

**TABLE 5.1 :** Percentage radioactivity associated with the various HPLC-derived peaks from extracts of leaf tissue of *L. esculentum* cv. Rodade.

| Time (days) | Radioactivity recovered per peak (%) | | | | | |
|---|---|---|---|---|---|---|
| | P1 (7-9 min) | P2 (37-39 min) | P3 (54-58 min) | P4 (62-63 min) | P5 (77-79 min) | P6 (80-81 min) |
| 1 | - | - | 4.0 | - | 90.4 | 5.6 |
| 5 | - | 30.4 | 4.4 | 15.2 | 45.5 | 4.5 |
| 10 | 0.4 | 40.1 | 13.4 | 12.0 | 30.7 | 3.4 |
| 15 | 0.3 | 41.7 | 25.0 | 12.5 | 18.3 | 2.2 |
| 20 | 0.2 | 46.4 | 21.2 | 17.0 | 12.9 | 2.3 |
| 25 | 0.2 | 47.4 | 10.2 | 30.1 | 10.0 | 2.1 |
| 30 | 0.3 | 44.1 | 9.0 | 37.0 | 7.1 | 2.5 |
| 35 | 0.3 | 34.1 | 8.3 | 49.0 | 5.9 | 2.4 |
| 40 | 0.3 | 24.7 | 7.8 | 60.7 | 5.7 | 0.8 |

### 5.3.2 Uptake and metabolism of $^{14}$C-BA by callus tissue of *L. esculentum*

In callus tissue of *L. esculentum* cv. Rodade a rapid uptake of $^{14}$C-BA was recorded during the 40 days of incubation (Figure 5.4). During the first 10 days, 66% of the total available BA was removed from the medium and was detected in the callus tissue. After 40 days of incubation on medium containing $^{14}$C-BA, 98% of all the available BA was detected in callus tissue of *L. esculentum*.

In the experiment a total of 6 HPLC-derived peaks were recorded in extracts from callus tissue of *L. esculentum*. The radioactive fractions co-eluted with [3G]BA in peak 1, [7G]BA in peak 2, [9R-MP]BA in peak 3, [9G]BA in peak 4, BA in peak 5 and [9R]BA in peak 6 (Table 5.2). Peak 3 co-chromatographed with authentic [9R-MP]BA, peak 5 with authentic BA and peak 6 with authentic [9R]BA following TLC. During the first day of incubation on medium containing $^{14}$C-BA a peak of radioactivity associated with the retention time of BA was the only peak recorded in extracts of callus tissue of *L. esculentum* cv. Rodade. A peak of radioactivity associated with [3G]BA was found in addition to BA after 5 days of exposure to $^{14}$C-BA, whereas an additional peak associated with the retention time of [9G]BA was detected after 10 days of culture.

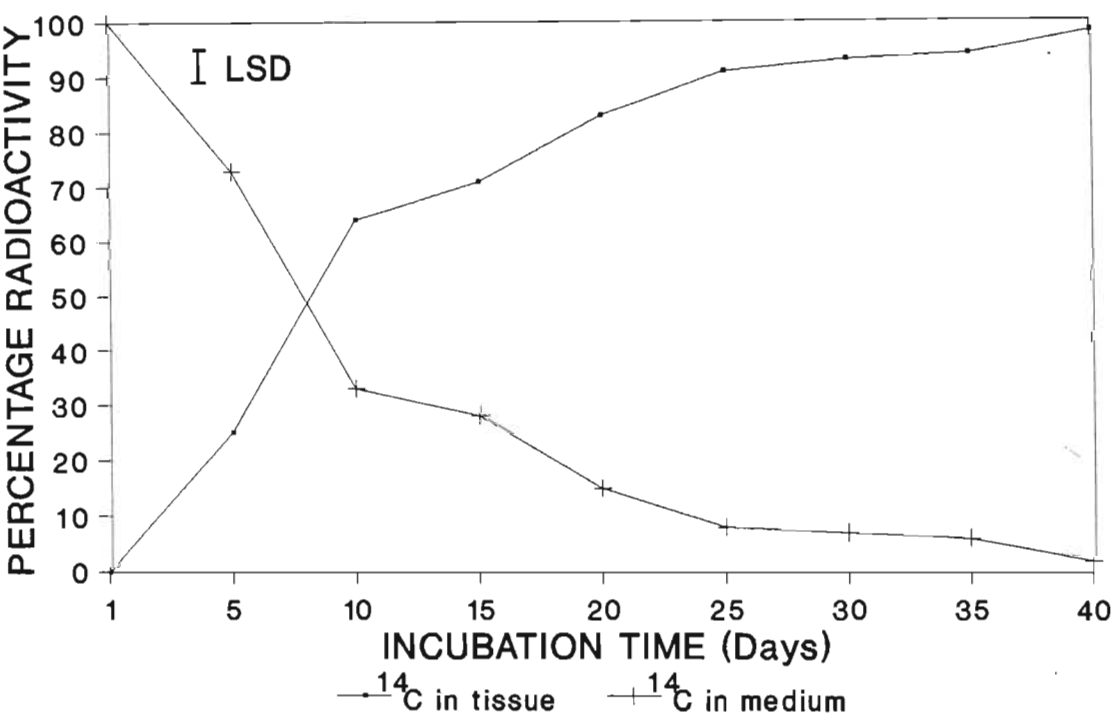


**FIGURE 5.4** : The uptake of $^{14}$C-BA over 40 days by callus tissue of *L. esculentum* cv. Rodade.

After 15 days of culture on a medium containing $^{14}$C-BA a fourth peak, associated with the retention time of [7G]BA was detected in addition to the previous 3 peaks. Peaks of radioactivity that co-eluted with [9R]BA (Peak 6) and [9R-MP]BA (Peak 3) were detected after 20 days of exposure to $^{14}$C-BA (Figure 5.5). This pattern persisted throughout the experiment. Between 20 and 40 days of exposure to $^{14}$C-BA, the peak associated with the retention time of [9R]BA showed a decrease in the percentage radioactivity associated with it. The peak that co-eluted with authentic [9R-MP]BA, increased over this period. The peak associated with BA decreased from the first day of incubation. The number of detectable BA metabolites increased up to day 20 after which it remained stable throughout the experiment. Peaks of radioactivity associated with the retention times of [3G]BA, [7G]BA and [9G]BA showed an increase in the amount of radioactivity during the course of the experiment.

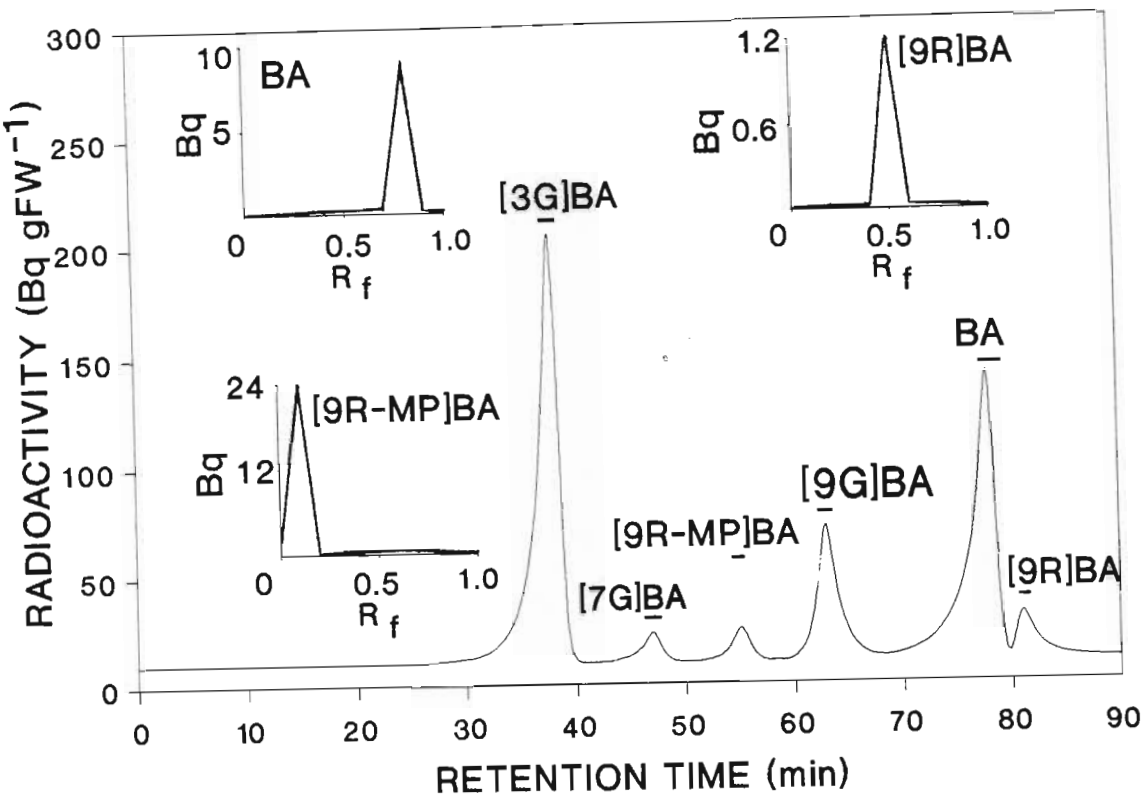


**FIGURE 5.5** : Radioactivity detected in an extract of callus tissue of *L. esculentum* cv. Rodade after 20 days of incubation on a medium containing $^{14}$C-BA. The retention time of authentic standards are indicated. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

**TABLE 5.2 :** Percentage radioactivity associated with the various HPLC-derived peaks from extracts of callus tissue of *L. esculentum* cv. Rodade.

| Time (days) | Radioactivity recovered per peak (%) | | | | | |
|---|---|---|---|---|---|---|
| | P1 (37-39 min) | P2 (47-48 min) | P3 (54-58 min) | P4 (62-63 min) | P5 (77-79 Min) | P6 (80-81 min) |
| 1 | - | - | - | - | 100.0 | - |
| 5 | 13.3 | - | - | - | 86.7 | - |
| 10 | 28.8 | - | - | 11.2 | 60.0 | - |
| 15 | 53.2 | 3.1 | - | 11.4 | 32.3 | - |
| 20 | 57.6 | 3.4 | 2.9 | 11.8 | 20.2 | 4.1 |
| 25 | 63.7 | 4.1 | 3.7 | 12.1 | 13.6 | 2.8 |
| 30 | 66.1 | 4.3 | 3.7 | 12.8 | 11.2 | 1.9 |
| 35 | 68.4 | 4.6 | 3.9 | 12.9 | 8.3 | 1.9 |
| 40 | 70.9 | 5.0 | 4.2 | 13.4 | 5.1 | 1.4 |

### 5.3.3 Uptake and metabolism of $^{14}$C-BA by leaf tissue of *L. peruvianum*

A sharp decrease in $^{14}$C-activity was recorded in the medium during the first 10 days of incubation on $^{14}$C-BA (Figure 5.6). A total of 79% of the available BA was removed from the medium during this period and was detected in the tissue. After 10 days the uptake of BA was slower. Leaf tissue of *L. peruvianum* accumulated 92% of the available radioactivity after 40 days of incubation.

In leaf tissue of *L. peruvianum* a total of 6 radioactive peaks could be detected following HPLC of the plant extracts (Table 5.3). Radioactivity co-eluted with authentic [3G]BA in peak 1, [7G]BA in peak 2, [9R-MP]BA in peak 3, authentic [9G]BA in peak 4, authentic BA in peak 5 and authentic [9R]BA inpeak 6. These radioactive peaks co-eluted with authentic standards (Figure 5.7). Following thin-layer chromatography, peak 3 co-chromatographed with [9R-MP]BA, peak 5 with BA and peak 6 with [9R]BA.

After one day of incubation, radioactive peaks associated with the retention times of authentic [9R-MP]BA, BA and [9R]BA were detected in extracts from leaf tissue of *L. peruvianum*. A peak associated with [3G]BA was detected in addition to the previous peaks after 10 days of incubation on a medium containing $^{14}$C-BA.

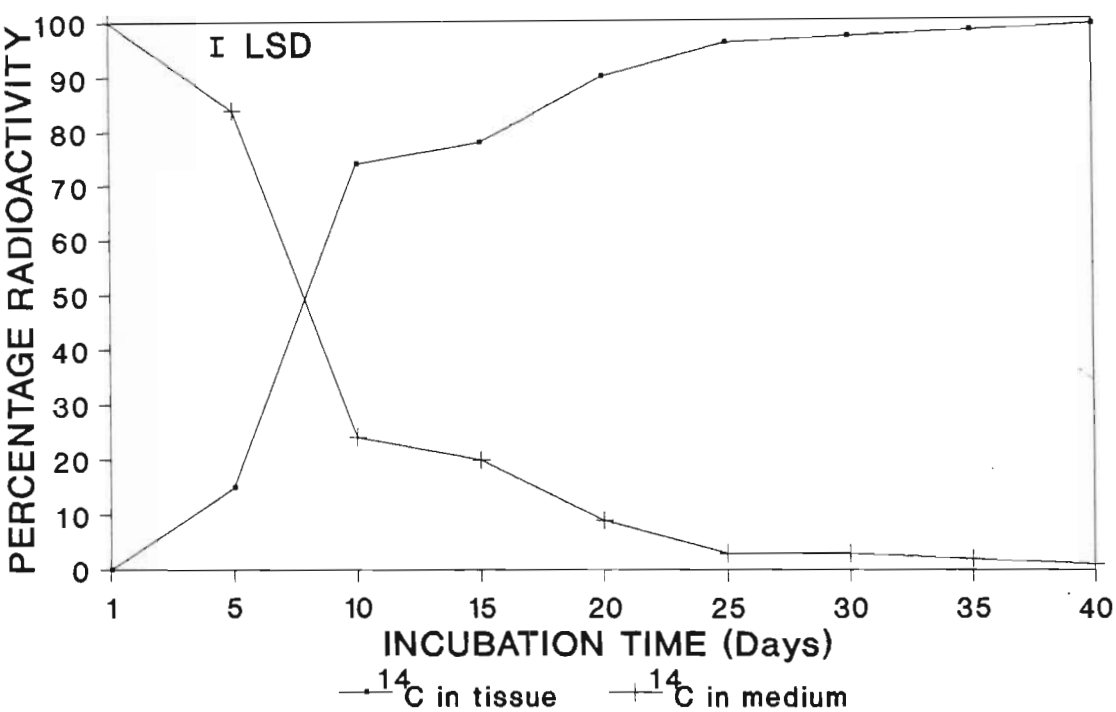


**FIGURE 5.6** : The uptake of $^{14}$C-BA by leaf tissue of *L. peruvianum*.

A total of 6 peaks were detected after 25 days of exposure when peaks of radioactivity associated with the retention times of [7G]BA and [9G]BA were detected in addition to the previous peaks detected. The percentage radioactivity of the peak that co-eluted with authentic BA decreased throughout the experiment. Peak 6, which co-eluted with [9R]BA, increased in radioactivity up to day 25 whereafter a decline in radioactivity was detected. The peak associated with [9R-MP]BA increased up to day 20 whereafter a slow decline in radioactivity was detected. Analysis of peaks associated with the glucosides of BA showed that [3G]BA reached an optimum after 20 days of incubation, that the peak co-eluting with [7G]BA reached an optimum after 30 days and that an optimal level was reached by the radioactive peak that co-eluted with[9G]BA after 35 days of incubation on medium containing $^{14}$C-BA.

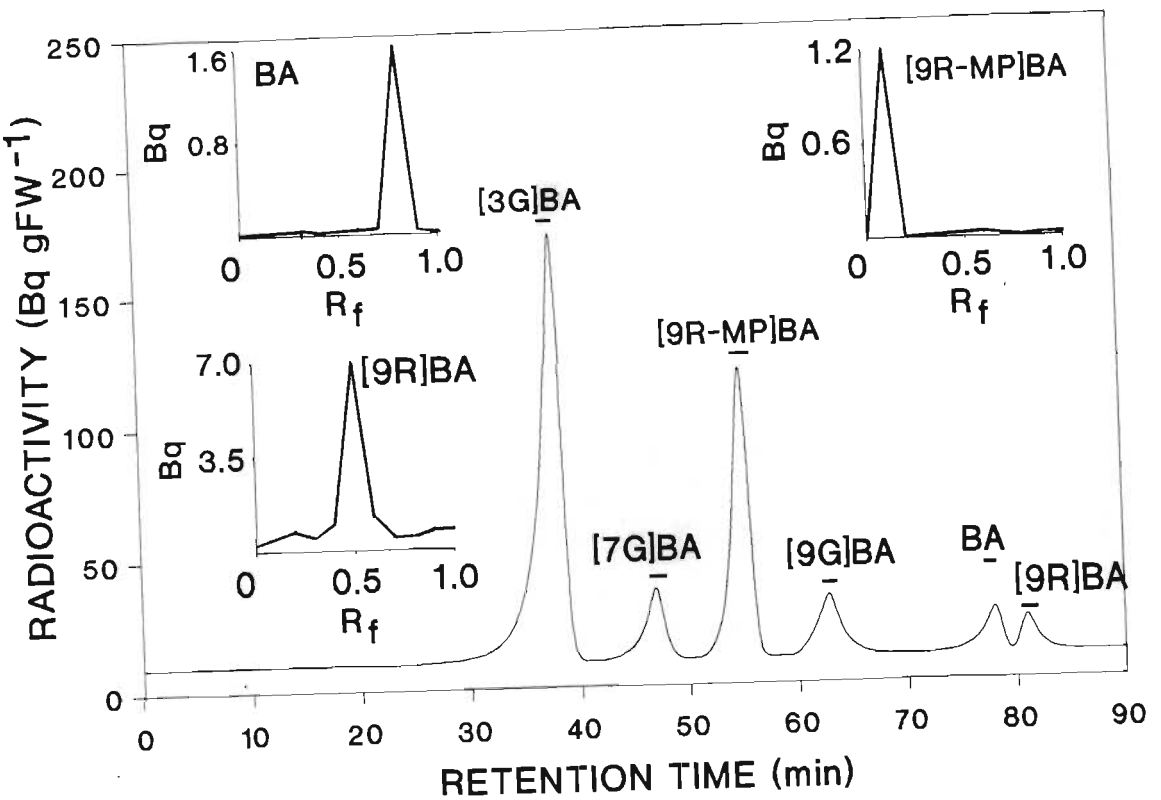


**FIGURE 5.7** : Radioactivity detected in an extract of leaf tissue of *L. peruvianum* after 25 days of incubation on a medium containing $^{14}$C-BA. The retention time of authentic standards are indicated. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

**TABLE 5.3 :** Percentage radioactivity associated with the various HPLC-derived peaks from extracts of leaf tissue of *L. peruvianum*.

| Time (days) | Radioactivity recovered per peak (%) | | | | | |
|---|---|---|---|---|---|---|
| | P1 (37-39 min) | P2 (47-48 min) | P3 (54-58 min) | P4 (62-63 min) | P5 (77-79 min) | P6 (80-81 min) |
| 1 | - | - | 10.5 | - | 86.7 | 2.8 |
| 5 | - | - | 21.3 | - | 74.3 | 4.4 |
| 10 | 21.1 | - | 22.5 | - | 51.1 | 5.3 |
| 15 | 48.6 | - | 23.0 | - | 22.4 | 6.0 |
| 20 | 55.7 | - | 22.3 | - | 15.6 | 6.4 |
| 25 | 49.0 | 6.9 | 21.9 | 5.6 | 10.1 | 6.5 |
| 30 | 31.6 | 17.5 | 21.0 | 18.3 | 6.4 | 5.2 |
| 35 | 25.2 | 21.5 | 20.4 | 22.8 | 5.1 | 5.0 |
| 40 | 20.1 | 26.5 | 20.1 | 24.6 | 4.0 | 4.7 |

### 5.3.4 Uptake and metabolism of $^{14}$C-BA by callus tissue of *L. peruvianum*

In callus tissue of *L. peruvianum* a linear uptake of $^{14}$C-BA was recorded during the course of the experiment (Figure 5.8). During this period a total of 94% of the available BA was removed from the medium and was subsequently detected in the callus tissue.

A total of 6 HPLC-derived radioactive peaks were recorded in extracts of callus tissue of *L. peruvianum* over the 40 days of incubation on medium containing $^{14}$C-BA (Table 5.4). These radioactive fractions co-chromatographed with authentic standards of [3G]BA in peak 1, [7G]BA in peak 2, [9R-MP]BA in peak 3, [9G]BA in peak 4, BA in peak 5 and [9R]BA in peak 6. These results were also supported by TLC where peaks 3, 5 and 6 co-chromatographed with [9R-MP]BA, BA and [9R]BA respectively. All 6 radioactive peaks were never found simultaneously at any incubation time throughout the experiment. Radioactive peaks associated with the retention times of BA and [9R]BA were recorded after one day of incubation. After 5 days of incubation on a medium containing $^{14}$C-BA a third peak of radioactivity that co-eluted with [9R-MP]BA was detected in the extract. Peaks of radioactivity associated with the retention time of [3G]BA was recorded after an incubation time of 10 and 15 days. The 7 glucoside of BA was added to the

radioactive spectrum of metabolites after 20 days of incubation (Figure 5.9). No more radioactivity associated with [3G]BA was detected after 20 days of culture. A new peak of radioactivity associated with the retention time of [9G]BA was however, detected after 25 days. Radioactivity associated with the retention time of [9R]BA was not detected after 40 days of culture. The percentage radioactivity recorded at the retention time of BA decreased during the experiment.

An optimum amount of radioactivity associated with [9R]BA was recorded after 25 days incubation. Levels of [9R-MP]BA increased throughout the experiment. The 3-,7- and 9 glucosides of BA were only produced during spesific periods of the experiment.

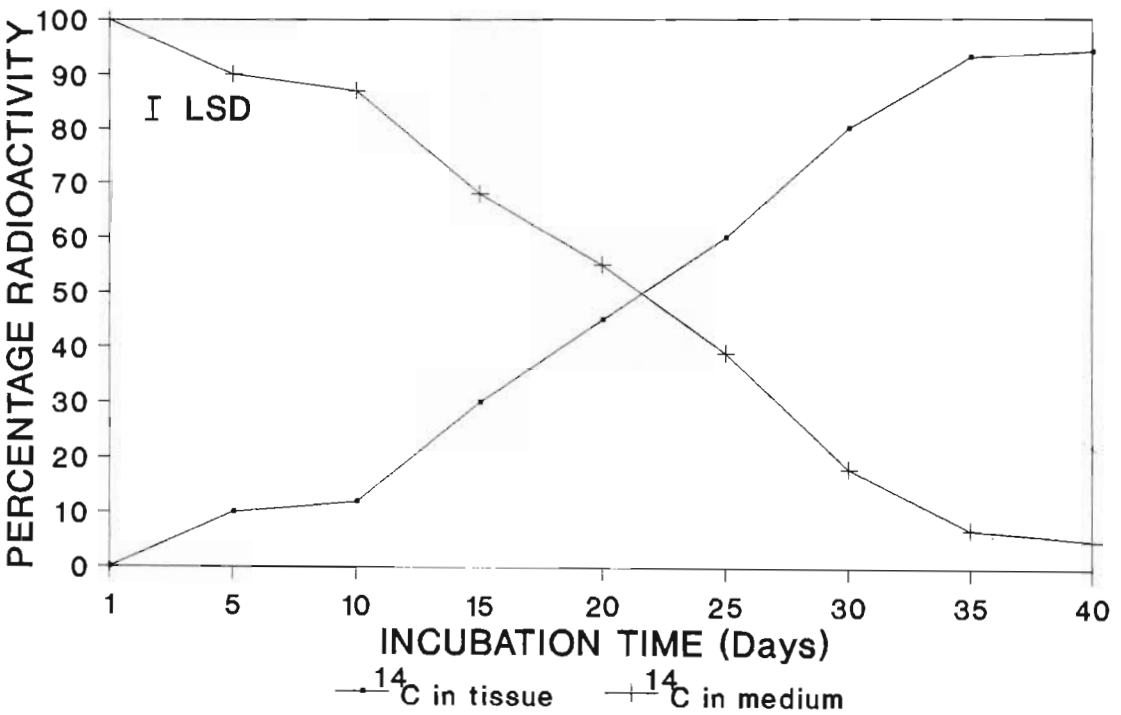


**FIGURE 5.8** : The uptake of $^{14}$C-BA over 40 days by callus tissue of *L. peruvianum*.

**TABLE 5.4 :** Percentage radioactivity associated with the various HPLC-derived peaks from extracts of callus tissue of *L. peruvianum*.

| | Radioactivity recovered per peak (%) | | | | | |
|---|---|---|---|---|---|---|
| Time (days) | P1 (37-39 min) | P2 (47-48 min) | P3 (54-58 min) | P4 (62-63 min) | P5 (77-79 min) | P6 (80-81 min) |
| 1 | - | - | - | - | 98.3 | 1.7 |
| 5 | - | - | 1.0 | - | 92.8 | 6.2 |
| 10 | 25.4 | - | 2.0 | - | 66.0 | 6.6 |
| 15 | 34.2 | - | 7.1 | - | 52.2 | 6.5 |
| 20 | 35.4 | 17.9 | 8.8 | - | 31.9 | 6.0 |
| 25 | - | 43.3 | 9.0 | 21.7 | 19.2 | 6.8 |
| 30 | - | 49.0 | 9.6 | 27.5 | 9.5 | 4.4 |
| 35 | - | 50.7 | 10.0 | 31.1 | 5.5 | 2.7 |
| 40 | - | 50.7 | 10.4 | 33.8 | 5.1 | - |

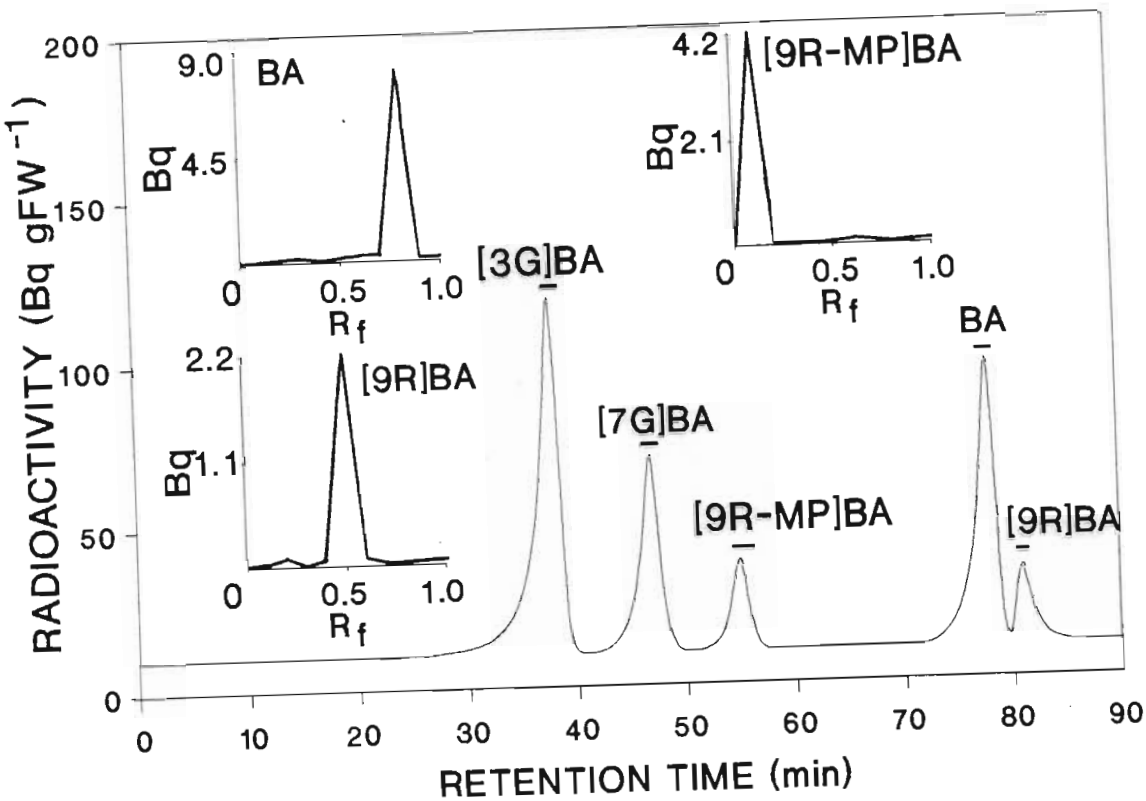


**FIGURE 5.9** : Radioactivity detected in an extract of callus tissue of *L. peruvianum* after 20 days of incubation on a medium containing $^{14}$C-BA. The retention time of authentic standards are indicated. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

## 5.4 DISCUSSION

The results indicated that BA uptake in leaf tissue of *L. esculentum* cv. Rodade and *L. peruvianum* occurred during the entire incubation period and that BA was taken up at a rate that could be described as linear in all four tissue types. This is in accordance with the findings of NORDSTROM and ELIASSON (1986) who found that the uptake of BA was linear in apple shoot cultures. The uptake in the tissues under investigation was slow but continuous over the whole experimental period. Similar result were reported by BLAKESLEY, LENTON and HORGAN (1991).

There was little loss of radioactivity in all four tissue types since all radioactivity removed from the medium could subsequently be detected in the tissue. Similar results was found by BLAKESLEY, LENTON and HORGAN (1991). BLAKESLEY and CONSTANTINE (1992) found in *Musa*, *Hippeastrum*, *Rhododendron*, *Gerbera jamesonii* and various other species that patterns of uptake between the different species were the same. These results were confirmed by this study.

The rate of BA uptake and the shooting response found for the different tissue types (Chapter 4) could be correlated. The shooting response might therefore well

be influenced by the availability of BA in the medium (BORNMAN and VOGELMANN, 1984; NORDSTROM and ELIASSON, 1986).

The term "metabolism" can be interpreted in a number of ways. VAN STADEN and DAVEY (1979) stated that with regard to cytokinin metabolism at least three metabolic routes should be considered. They showed that cytokinins could be incorporated into other molecules, that they may be broken down by catabolic processes and be destroyed, and that they may thirdly be converted to inactive storage forms. These storage compounds may be converted to active forms of the cytokinin under certain conditions. VAN STADEN and BAYLEY (1990) found in *Phaseolus vulgaris* that roots metabolised BA the most extensively and that stem as well as leaf tissue absorbed less BA than roots, but that metabolism in these tissue types was however, more rapid, suggesting that all plant organs do not metabolize cytokinins at the same rate nor to the same extent. The results with callus and leaf tissue of *L. esculentum* and *L. peruvianum* confirms these findings. There was, however, more similarities between different tissue types of the tomato species than was the case for *Phaseolus*. The results also suggest that the main route of BA metabolism is from BA to [9R]BA and that [9R-MP]BA is produced from [9R]BA. These results are also in accordance with the interpretation of VAN STADEN and BAYLEY (1990).

A great deal of interconversion was found between BA, [9R]BA and [9R-MP]BA in both tissue types of *L. esculentum* and *L. peruvianum*. This was also reported by VAN STADEN and BAYLEY (1991) for tomato shoots. However, some interconversion was found between the main metabolic route and adenine in leaf tissue of *L. esculentum*. BLAKESLEY, LENTON and HORGAN (1991) found in *Gerbera jamesonii* that [9R]BA accumulated in callus tissue whereas the 3-glucoside accumulated in leaf tissue. During a study of the metabolism of BA in *Musa* and *Rhododendron* it was shown that [9G]BA was the principal metabolite formed in the tissue (BLAKESLEY and CONSTANTINE, 1992). In this study it was found that no unique metabolite could be identified in any tissue type. However, in both tissue types of *L. peruvianum* [7G]BA was produced whereas it was found only in callus tissue of *L. esculentum*. In both tissue types of *L. esculentum* the 3 and 9-glucosides were produced to a larger extent than in tissue of the wild species. It was shown that there was considerable similarities between the two species studied, however tissue types were very different.

The results of this study also show that callus tissue of *L. esculentum* convert BA to the 3 and 9-glucosides and that much less [9R-MP]BA is detected in the tissue. The other tissue types studied, convert BA to [9R-MP]BA, [9R]BA or [7G]BA which constitutes the active forms of the cytokinin related to shoot initiation and

proliferation (VAN STADEN and DAVEY, 1979; BLAKESLEY, LENTON and HORGAN, 1991). In this might also lie the key to the lack of morphogenesis in callus tissue of the tomato. The system for the conversion from BA to [9R-MP]BA seems to be slow, hence causing an inability to produce adventitious shoots *in vitro*. On the other hand, the system to convert BA to its 3 and 9-glucosides may simply be better developed in the tomato causing an accumulation of the 3 and 9-glucosides as storage products (VAN STADEN and DAVEY, 1979). The system for converting these glucosides to active forms of the cytokinin might also be inoperative.

It is evident that the uptake of BA is a critical factor in the induction of adventitious shoots in all four tissue types. The availability of BA may play an important role in morphogenesis. This study has shown that the uptake of BA in callus tissue of *L. esculentum* is in fact much faster than what was expected. Shortened subculture periods might help to optimize conditions for the regeneration of adventitious shoots on callus tissue of the tomato.

The high rate of production of the 3-, 7- and 9-glucosides in callus tissue of tomato could also be a factor preventing this tissue type to regenerate adventitious shoots, since these compounds constitute the inactive forms of the cytokinin and are often

viewed as storage metabolites (MCGAW, 1987). The ability to convert them back to active forms also seems to be underdeveloped and it would seem that after BA is converted to these two glucosides, they do not contribute to the initiation of adventitious buds *in vitro*. The second problem is with the low production of [9R-MP]BA. Since this compound is one of the active forms of the cytokinin (LETHAM and PALNI, 1983; BAYLEY, VAN STADEN, MALLETT and DREWES, 1989), its endogenous concentration might not be high enough to induce the production of adventitious shoots.

This study indicated that differences and possible inoperative pathways in the metabolism of BA in callus tissue of *L. esculentum* might play an important role in the initiation of adventitious shoots *in vitro* since it was found that the BA glucosides are the major metabolic forms produced in callus tissue of the tomato cultivar under investigation and that the active forms of the cytokinin are produced at low concentrations in this tissue type.

# CHAPTER 6

# THE EFFECT OF AUXIN CONCENTRATION ON THE METABOLISM OF 6-BENZYLADENINE AFTER 10 DAYS OF INCUBATION ON $^{14}$C-BA.

## 6.1 INTRODUCTION

Cytokinins, first recognised for their ability to induce cell division *in vitro* are now known to induce a diversity of responses in plants. The regulation of growth, differentiation and plant development involves complex interactions between two or more growth regulators (WAREING, 1977). The influence of auxins and cytokinins in the control of morphogenesis in cultured cells and explants is also well known (LAMPUGNANI, FANTELLI, LONGO, LONGO and ROSSI, 1981). As early as 1948, SKOOG and TSUI found that the inhibitory effect of auxin on shoot initiation could be reversed by the addition of adenine sulphate. These studies led to the hypothesis that organogenesis is controlled by a balance between cytokinins and auxins (SKOOG and MILLER, 1957). It is also evident that

exogenously supplied cytokinins and auxins have an influence on endogenous levels of growth regulators and that the endogenous balance of these compounds have an effect on morphogenesis (DODDS and ROBERTS, 1985). However, the biochemical and molecular basis of this interaction is not known. An understanding of the processes involved is therefore necessary in order to understand how to modify endogenous levels of these compounds and in so doing, to influence plant development *in vitro*.

PALNI, BURCH and HORGAN (1988) studied the effect of NAA concentration on the stability of zeatin riboside using the tobacco stem pith system. Their results indicated that cytokinin stability is inversely related to auxin concentration, an effect which may be partly mediated through cytokinin oxidase. In plant tissues, cleavage of the side chains of the natural cytokinins zeatin and iP as well as their ribosides are attributable to this enzyme system (LETHAM and PALNI, 1983). However, the analogous degradation of the synthetic cytokinin BA, cannot be explained by this system which means that plant species must contain an enzyme system distinct from cytokinin oxidase, which cleaves benzyl groups from the $N^6$ position. This aspect needs further clarification, particularly in view of the recent report that BA seems to occur naturally in plant tissue (NANDI, LETHAM, PALNI, WONG and SUMMONS, 1989). In extracts of radish cotyledons an

enzyme system, cytokinin 7-glucosyl transferase, was detected which converted BA into its 7 and 9-glucosides (ENTSCH and LETHAM, 1979).

In this study the effect of IAA concentration on the metabolism of BA was investigated in callus and leaf tissue of *L. esculentum* and *L. peruvianum* to establish whether or not there are differences in the metabolism of BA in the different tissue types. This study together with the previous study on the uptake and metabolism of BA could help to clarify the possible effect that IAA may have on the metabolism of BA in the tissue types under investigation. This may help in our understanding of the interactions between cytokinins and auxins in plant morphogenesis.

## 6.2 MATERIALS AND METHODS

### 6.2.1 The production of leaf material

Leaf material were produced and cultured under conditions described earlier (Chapter 3).

### 6.2.2 The production of callus

Callus tissue were produced and cultured as described earlier.

### 6.2.3 Preparation of material for experiments

Plant material was prepared as described earlier for the uptake and metabolism of BA.

### 6.2.4 Preparation of medium

In this study 8-$^{14}$C-benzyladenine with a specific activity of 1.85 MBq was added to an MSO medium to give an approximate activity of 81.5 kBq in each 10 $cm^3$ aliquot of medium. Unlabelled BA was also added to the medium to ensure the appropriate BA concentrations necessary for the growth of the different tissue types (Table 3.4). Indole-3-acetic acid (IAA) was added to the different media in the range 0.0, 0.1, 1.0, 2.0, 3.0 and 4.0 mg $dm^{-3}$ in order to determine the effect of different IAA concentrations on the metabolism of BA.

### 6.2.5 Incubation of explants

The four different tissue types (leaf and callus tissue of *L. esculentum* and *L. peruvianum*) were incubated on the appropriate media containing BA for a period of 10 days. At the end of the incubation time the material was removed from the medium, frozen and subsequently extracted for cytokinins.

### 6.2.6 Extraction of cytokinins

Cytokinins were extracted from leaf and callus tissue of *L. esculentum* cv. Rodade and *L. peruvianum* as described earlier (Chapter 5).

### 6.2.7 Determination of $^{14}$C-BA uptake

To determine the radioactivity remaining in the media as well as in the plant tissue, methods were used as described earlier (Chapter 5).

Twenty samples of each treatment were analysed in all tissue types tested. The sum, mean and standard deviation were determined and the least significant difference (LSD) ($p<0.01$) calculated.

### 6.2.8 HPLC analysis of extracts

The extracts were fractionated by HPLC using the methods as described earlier (Chapter 5).

### 6.2.9 Determination of radioactivity

Each fraction recovered by HPLC was scanned for radioactivity as described earlier (Chapter 5). A total of four separate analysis were performed for each tissue type.

### 6.2.10 TLC analysis of compounds

A TLC analysis of HPLC fractions associated with the retention time of BA, [9R]BA and [9R-MP]BA were performed according to methods described earlier (Chapter 5).

## 6.3 RESULTS

### 6.3.1 Leaf tissue of *L. esculentum*

In leaf tissue of *L. esculentum* cv. Rodade radioactive peaks that co-chromatographed with authentic standards of [3G]BA, [9R-MP]BA, BA and [9R]BA were detected at a concentration of 0.0 mg $dm^3$ IAA (Figure 6.1). The tissue produced additional peaks associated with the retention times of Ade and [9G]BA at IAA concentration between 1.0 and 4.0 mg $dm^{-3}$. Concentrations of IAA higher than 1.0 mg $dm^{-3}$ produced all six radioactive peaks throughout the experiment (Figure 6.2). Following TLC the radioactive peaks associated with the retention times of [9R-MP]BA, BA and [9R]BA co-chromatographed with authentic standards of [9R-MP]BA, BA and [9R]BA respectively.

The peak associated with the retention time of Ade showed an increase in radioactivity from 0.1 to 4.0 mg $dm^{-3}$ IAA. A much greater increase in levels of radioactivity was detected for the peak that co-chromatographed with authentic [3G]BA at IAA concentrations from 0.0 to 4.0 mg $dm^{-3}$. Levels of radioactivity associated with the retention time of [9R-MP]BA decreased with increasing IAA concentration whereas radioactivity associated with the retention time of authentic

[9G]BA increased from 1.0 to 4.0 mg $dm^{-3}$ IAA. This peak was not detected at concentrations below 1.0 mg $dm^{-3}$ IAA.

The radioactive peak that co-chromatographed with authentic BA showed a decrease in associated radioactivity with increasing concentrations of IAA. The same situation occurred for the peak associated with the retention time of [9R]BA. The percentage $^{14}C$-BA recovered from the tissue showed a gradual increase with increasing IAA concentration (Figure 6.3).

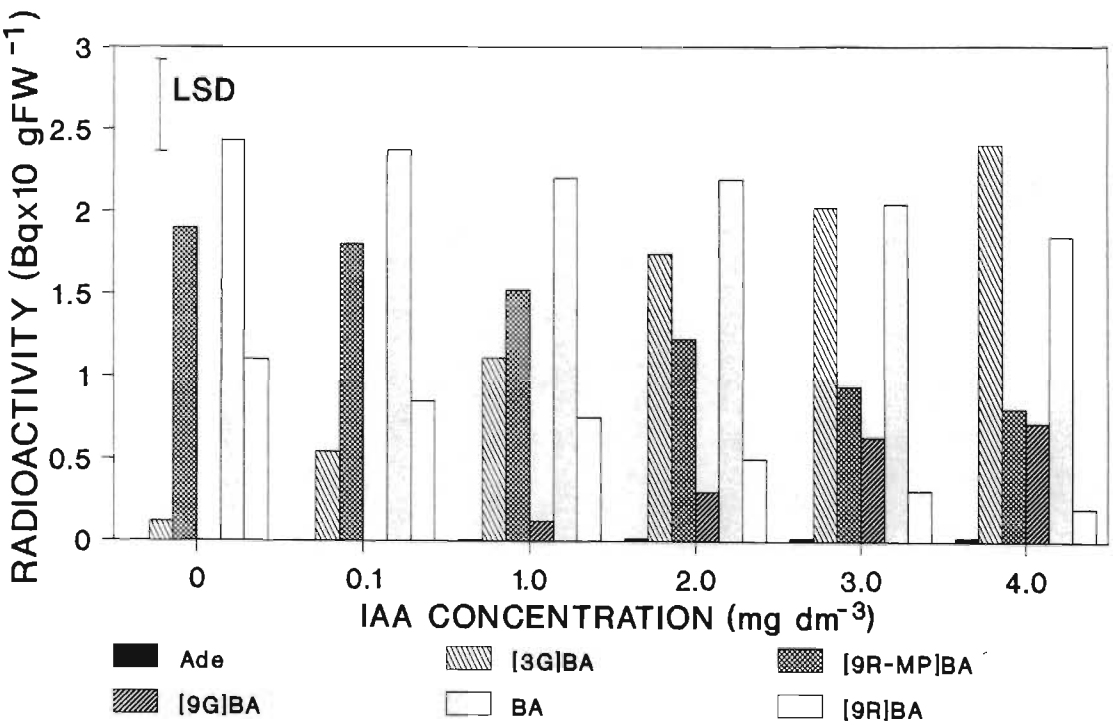


**FIGURE 6.1** : Peaks of radioactivity detected in leaf tissue of *L. esculentum* cv. Rodade after 10 days of incubation on media containing $^{14}$C-BA and six different concentrations of IAA.

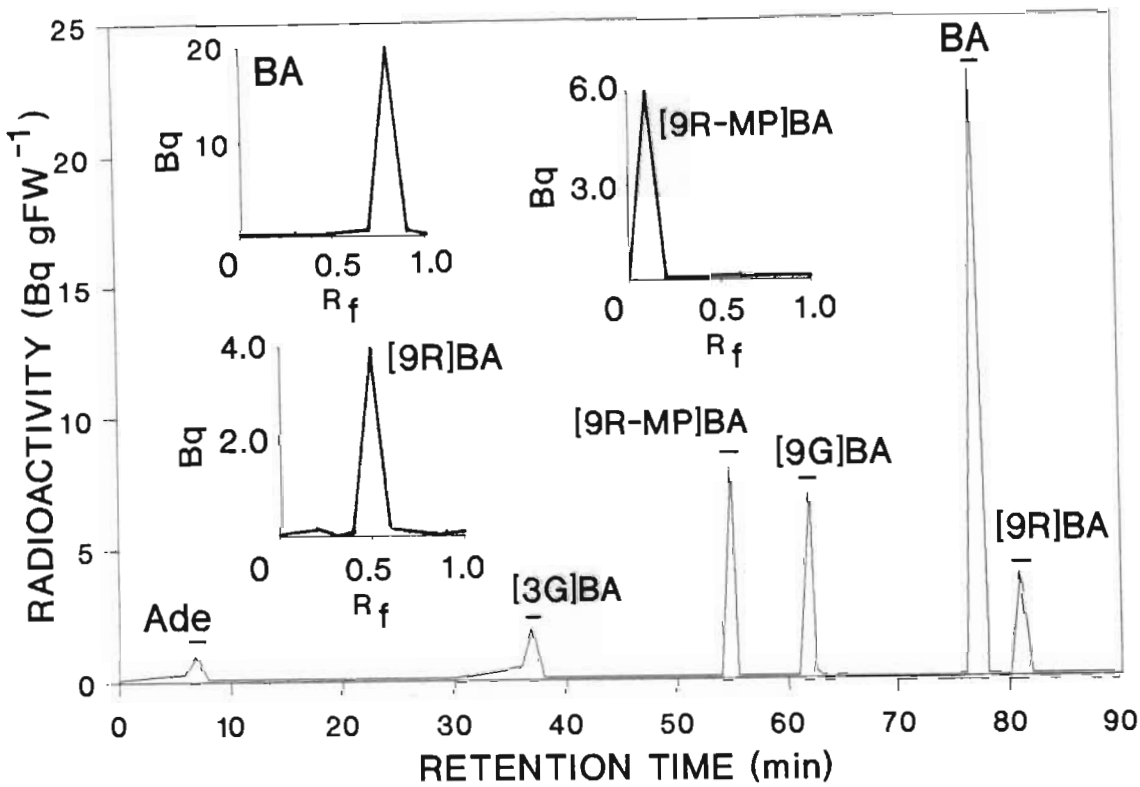


**FIGURE 6.2** : Radioactivity detected in an extract of leaf tissue of *L. esculentum* cv. Rodade after 10 days of incubation on a medium containing $^{14}$C-BA and 3.0 mg dm$^{-3}$ IAA. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

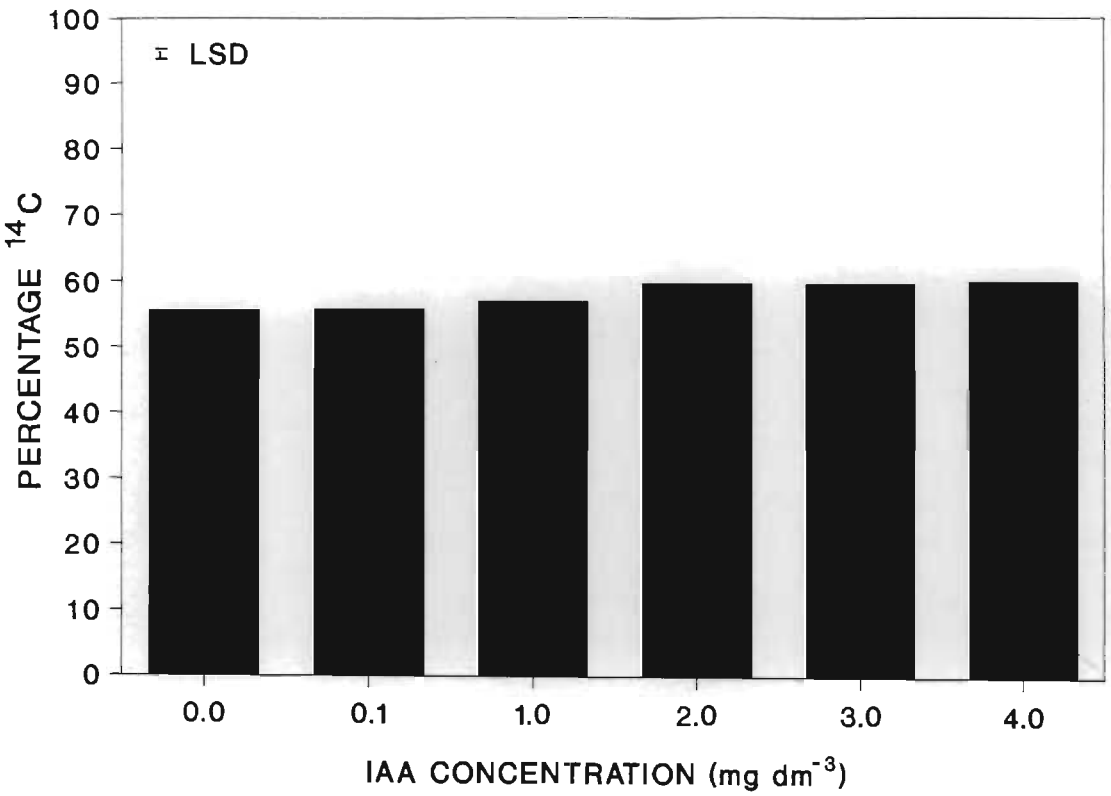


**FIGURE 6.3** : Percentage radioactivity ($^{14}$C-BA) recovered from leaf tissue of *L. esculentum* cv. Rodade after 10 days of culture in the presence of $^{14}$C-BA using six different concentrations of IAA.

### 6.3.2 Callus tissue of *L. esculentum*

Extracts of callus tissue of *L. esculentum* cv. Rodade yielded three radioactive peaks after 10 days of culture on $^{14}$ C-BA (Figure 6.4). The first peak co-eluted with [3G]BA, the second with [9G]BA and the third with authentic BA following HPLC separation of the extracts (Figure 6.5). The third peak also co-chromatographed with an authentic standard of BA folowing TLC.

The radioactivity associated with the peak that co-chromatographed with authentic [3G]BA remained constant at all IAA concentrations and no significant differences could be detected between different treatments. Radioactivity associated with the retention time of [9G]BA also showed no significant differences between the different treatments. The same trend was observed in the radioactivity that co-eluted with authentic BA. There were no significant differences in the percentage radioactivity recovered from the tissue at different IAA concentrations (Figure 6.6).

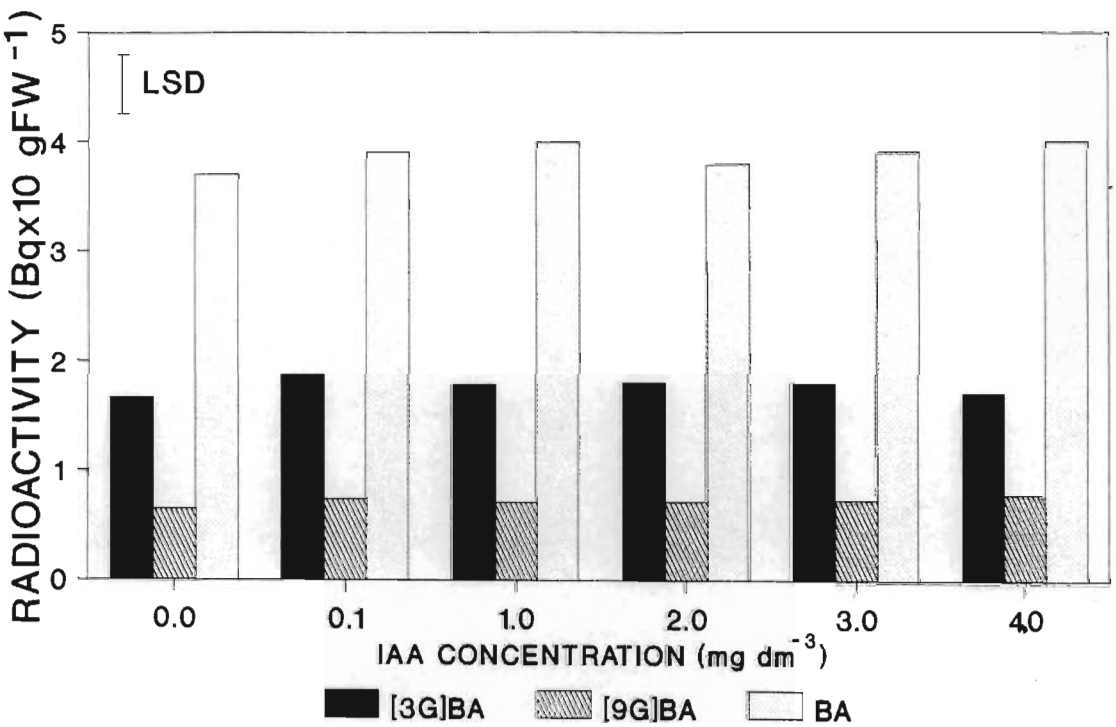


**FIGURE 6.4** : Peaks of radioactivity detected in callus tissue of *L. esculentum* cv. Rodade after 10 days of incubation on media containing $^{14}$C-BA and six different concentrations of IAA.

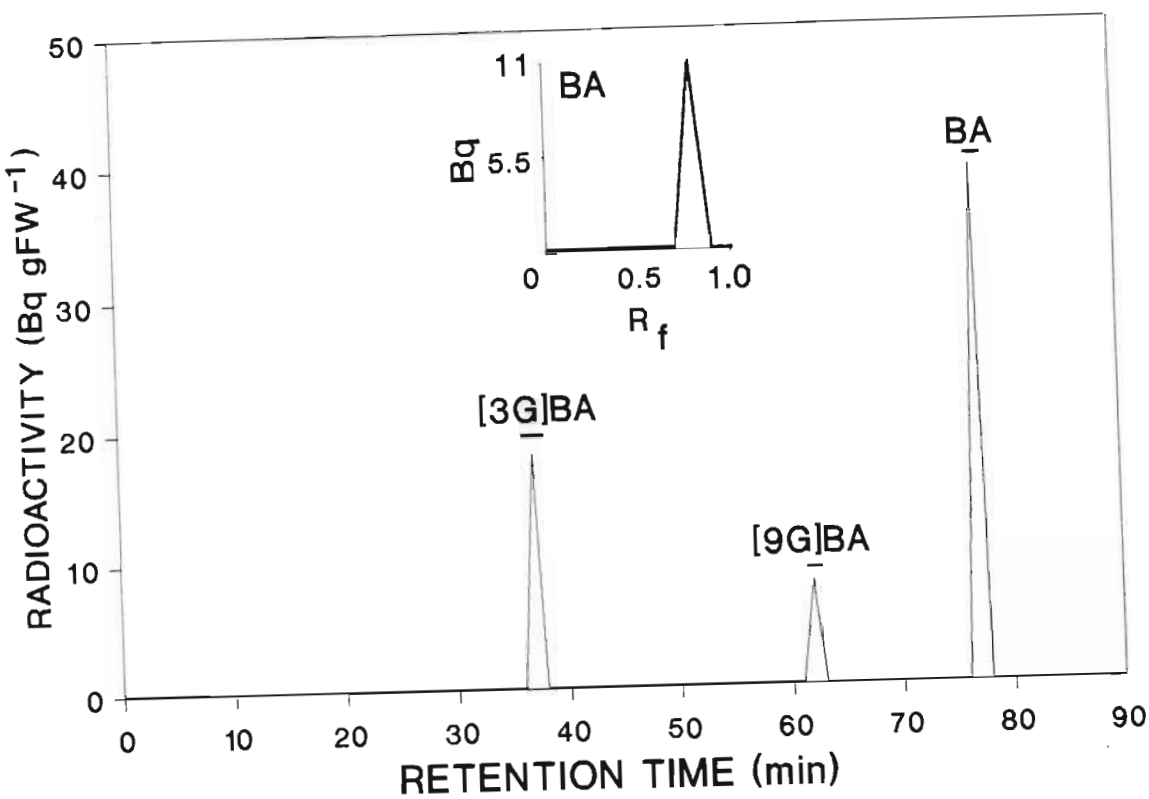


**FIGURE 6.5** : Radioactivity detected in an extract of callus tissue of *L. esculentum* cv. Rodade after 10 days of incubation on a medium containing $^{14}$C-BA and 3.0 mg dm$^{-3}$ IAA. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

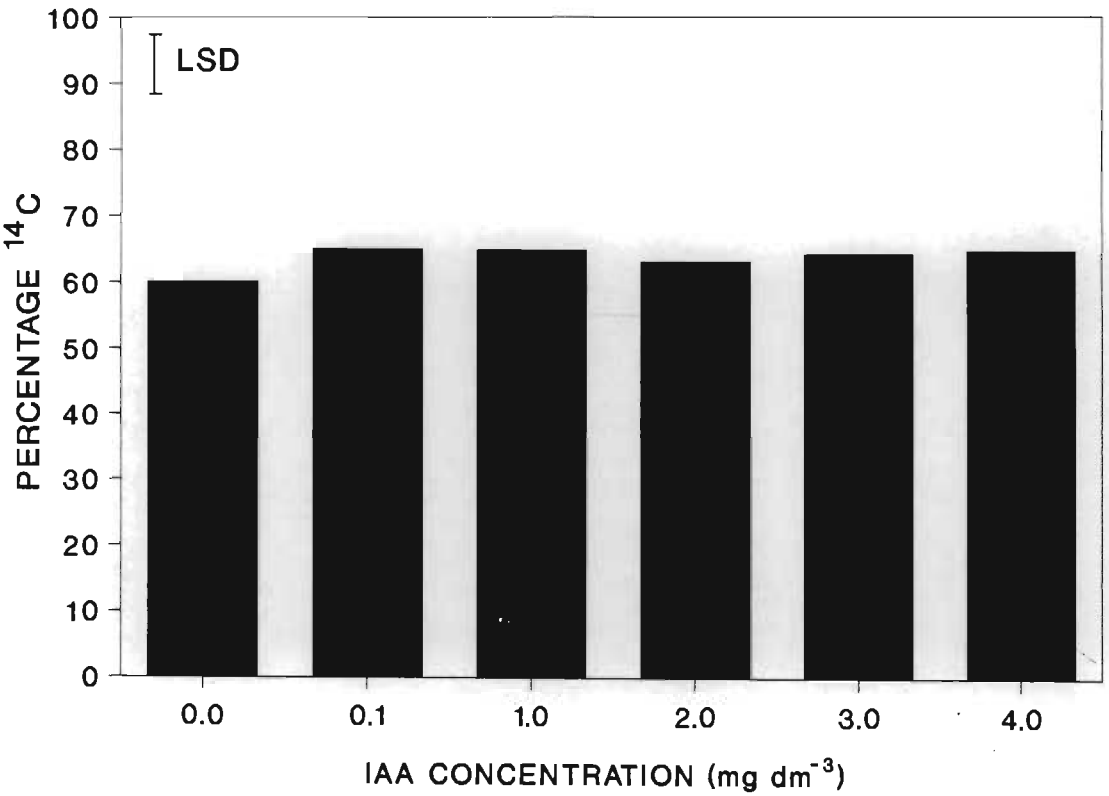


**FIGURE 6.6** : Percentage radioactivity ($^{14}$C-BA) recovered from callus tissue of *L. esculentum* cv. Rodade after 10 days of culture in the presence of $^{14}$C-BA using six different concentrations of IAA.

### 6.3.3 Leaf tissue of *L. peruvianum*

A total of 4 HLPC-derived radioactive peaks were recovered from extracts of leaf tissue of *L. peruvianum* after 10 days of culture on $^{14}$C-BA at all of the IAA concentrations tested (Figure 6.7). During HPLC the first peak co-chromatographed with authentic [3G]BA, the second with [9R-MP]BA, the third with BA and the fourth with [9R]BA (Figure 6.8). Following thin-layer chromatography the second HPLC-derived radioactive peak co-chromatographed with authentic [9R-MP]BA, the third with BA and the fourth radioactive peak with an authentic standard of [9R]BA.

In leaf tissue of *L. peruvianum* the peak of radioactivity associated with the retention time of [3G]BA showed an increase in activity with increasing IAA concentration in the medium. The peaks of radioactivity that co-eluted with authentic [9R-MP]BA, BA and [9R]BA showed a decrease in the levels of radioactivity during an increase in the IAA concentration. In this tissue type radioactivity associated with BA remained at high levels throughout the experiment. The percentage radioactivity recovered from the tissue increased gradually with increasing IAA concentrations (Figure 6.9).

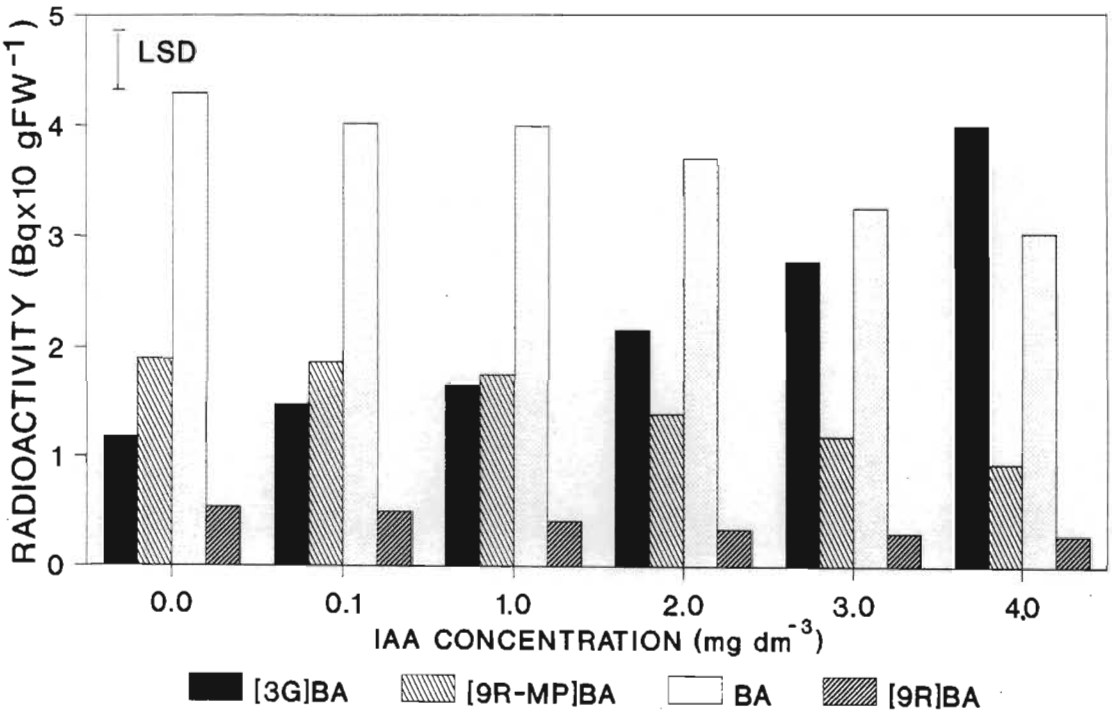


**FIGURE 6.7** : Peaks of radioactivity detected in leaf tissue of *L. peruvianum* after 10 days of incubation on media containing $^{14}$C-BA and six different concentrations of IAA.

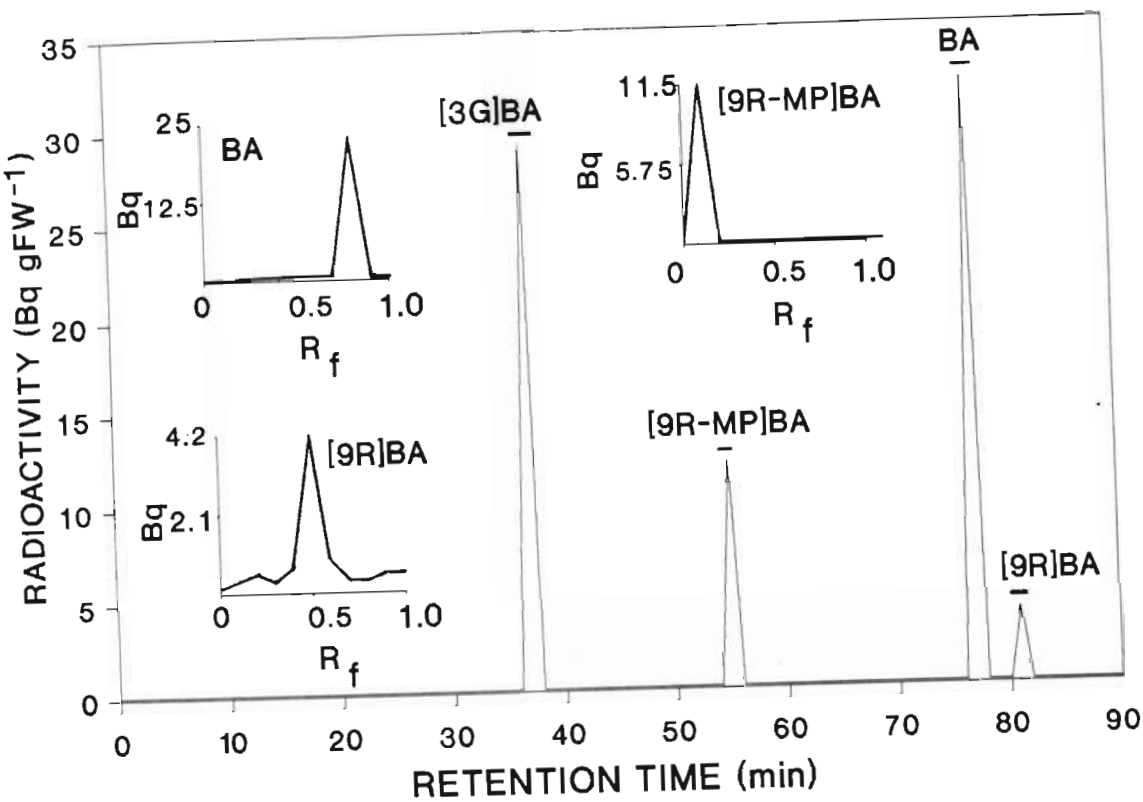


**FIGURE 6.8** : Radioactivity detected in an extract of leaf tissue of *L. peruvianum* after 10 days of incubation on a medium containing $^{14}$C-BA and 3.0 mg dm$^{-3}$ IAA. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

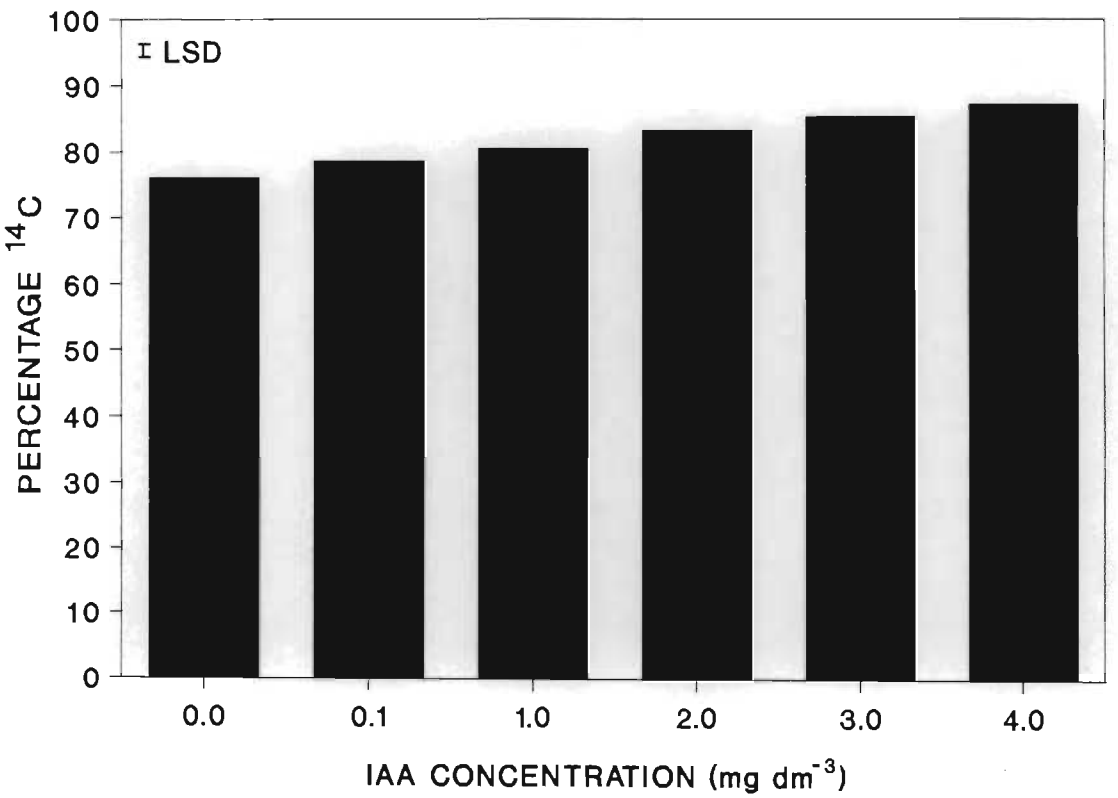


**FIGURE 6.9** : Percentage radioactivity ($^{14}$C-BA) recovered from leaf tissue of *L. peruvianum* after 10 days of culture in the presence of $^{14}$C-BA using six different concentrations of IAA.

### 6.3.4 Callus tissue of *L. peruvianum*

The callus tissue of *L. peruvianum* yielded four peaks of radioactivity following HLPC separation of the extracts after 10 days of culture on $^{14}$C-BA (Figure 6.10). The radioactive peaks co-chromatographed with authentic standards of [3G]BA, [9R-MP]BA, BA and [9R]BA. Peaks of radioactivity associated with [9R-MP]BA, BA and [9R]BA co-chromatographed with authentic standards of these compounds following thin-layer chromatography (Figure 6.11).

The peak of radioactivity associated with the retention time of [3G]BA showed an increase in activity with increasing IAA concentration in the medium. Radioactive peaks that co-chromatographed with [9R-MP]BA, BA and [9R]BA showed a decrease in radioactivity with increasing IAA concentration. The percentage radioactivity recovered from callus tissue of *L. peruvianum* showed no increase in radioactivity from 0.0 to 2.0 mg $dm^{-3}$ IAA. Concentrations of IAA from 3.0 to 4.0 mg $dm^{-3}$ caused a significant increase in the amount of radioactivity recovered from the tissue (Figure 6.12).

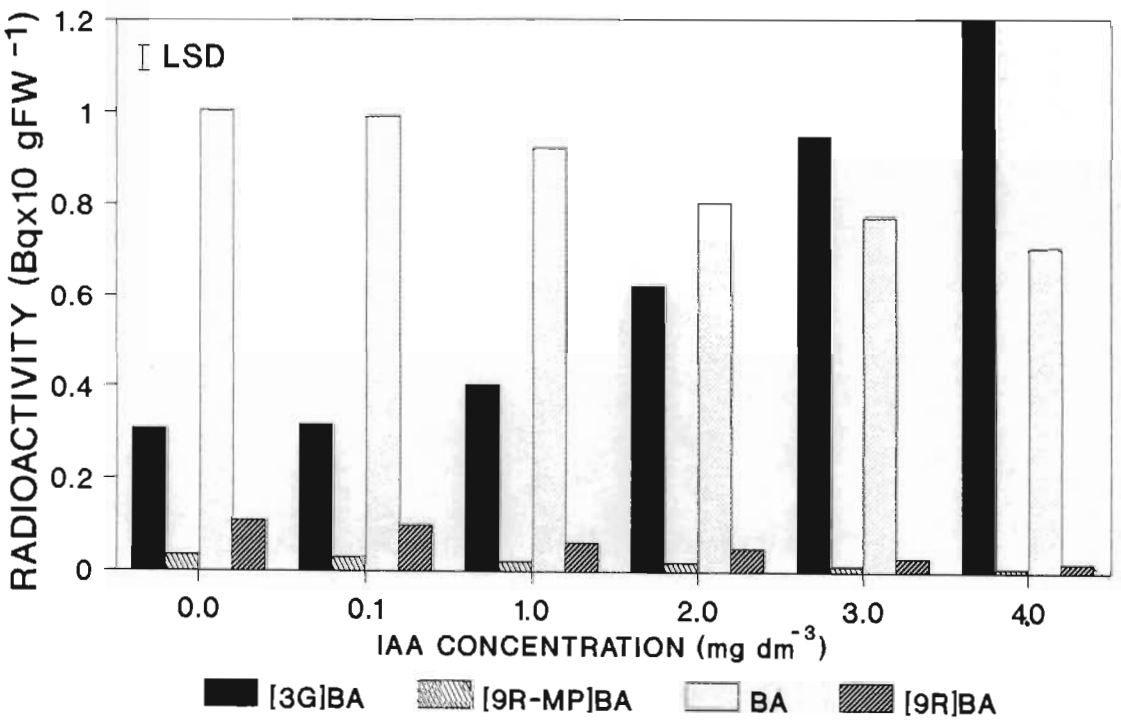


**FIGURE 6.10** : Peaks of radioactivity detected in callus tissue of *L. peruvianum* after 10 days of incubation on media containing $^{14}$C-BA and six different concentrations of IAA.

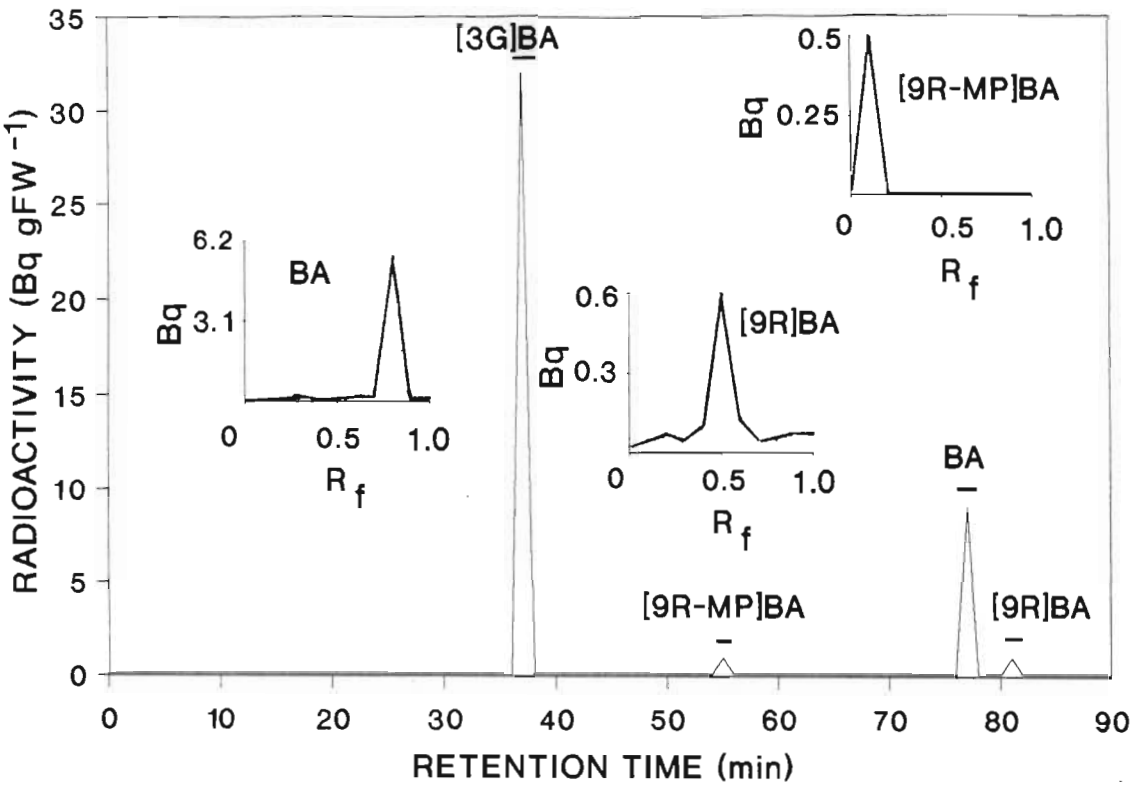


**FIGURE 6.11** : Radioactivity detected in an extract of callus tissue of *L. peruvianum* after 10 days of incubation on a medium containing $^{14}$C-BA and 3.0 mg dm$^{-3}$ IAA. Inserts represent TLC chromatograms of aliquots of specific peaks of radioactivity as indicated by captions.

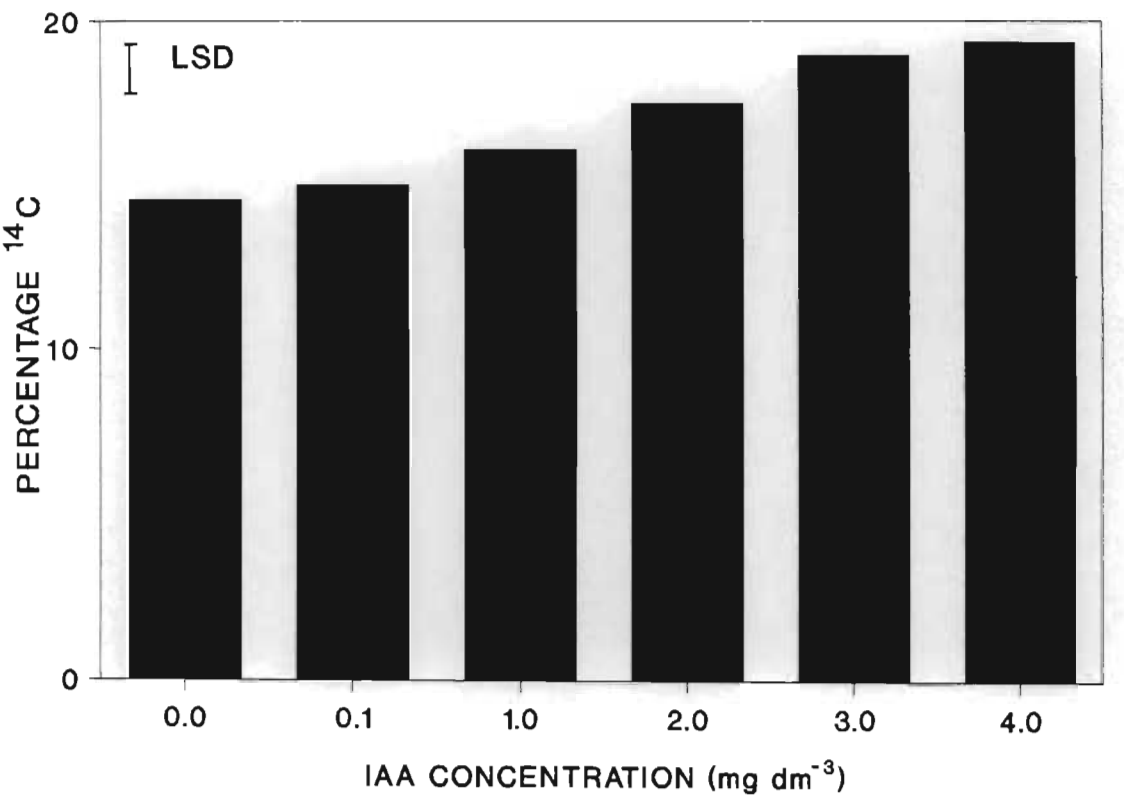


**FIGURE 6.12** : Percentage radioactivity ($^{14}$C-BA) recovered from callus tissue of *L. peruvianum* after 10 days of culture in the presence of $^{14}$C-BA using six different concentrations of IAA.

## 6.4 DISCUSSION

Previous studies with tomato organs (BAYLEY, VAN STADEN, MALLETT and DREWES, 1989) and *Phaseolus vulgaris* (VAN STADEN and BAYLEY, 1990) have indicated that differences in the metabolism of BA exists in different organs of the same species. This study have shown that BA is metabolized to a greater extent in leaf tissue than in callus tissue of *L. esculentum* cv Rodade under the conditions tested. However, in leaf and callus tissue of *L. peruvianum* the same metabolites are found. The number of metabolites as well as the percentage radioactivity in these experiments was in accordance with the findings on the uptake of BA in chapter 5 for both tissue types of the two species.

PALNI, BURCH and HORGAN (1988) found a cytokinin oxidase system to be partly controlled by NAA concentration and that higher NAA concentrations caused more zeatin riboside to be converted to Ade, Ado and adenosine nucleotides. Although the same enzyme system is probably not causing the breakdown of BA in leaf and callus tissue of *L. esculentum* the same trend was noted in these tissues. This result needs more investigation since BA was found in plant tissue by NANDI, LETHAM, PALNI, WONG and SUMMONS (1989) which makes the presence of a similar enzyme system a strong possibility. A radioactive peak associated with

the retention time of Ade was found in leaf tissue of *L. esculentum*. The conversion of BA to the 3- and 9-glucosides of BA could indicate on the presence of a cytokinin glucosyltransferase system similar to the one described by ENTSCH and LETHAM (1979). It was clear from these results that IAA concentration played a definite role in the conversion of BA to [3G]BA and [9G]BA in leaf tissue of *L. esculentum* and that IAA concentration had almost no effect on the same metabolic process in callus tissue of this tomato cultivar.

Levels of radioactivity that co-chromatographed with BA, [9R]BA and [9R-MP]BA decreased with increasing IAA concentration which suggests that IAA might cause a conversion of BA and its metabolites to the 3- and 9-glucosides of BA in leaf tissue of *L. esculentum* cv. Rodade. This system is either inactive or non-existent in callus tissue of this tomato cultivar since both [9R]BA and [9R-MP]BA were neither detected in extracts from callus tissue nor was the concentration of these peaks influenced by any concentration of IAA.

In leaf and callus tissue of *L. peruvianum* a similar spectrum of metabolites were detected for both tissue types. In both tissue types of this species it was also found that cytokinin stability was influenced by IAA concentration. The percentage radioactivity recovered as well as the metabolites correlated well with results on the

uptake of BA found in chapter 5. In these tissue types peaks of radioactivity associated with [3G]BA, [9R-MP]BA, BA and [9R]BA were detected.

The results of this study are of considerable importance since it emphasizes that IAA interacts with BA in a complex manner to control the endogenous levels of BA and its metabolites. This also stresses the importance of auxins in regulating cytokinin metabolism as well as their endogenous levels (HANSEN, MEINS and MILANI, 1985). The significance of this regulation with regard to the cytokinin metabolites produced and their role in organogenesis is somewhat obscure. A definite factor in this process seems to be the metabolism and catabolism of [9R-MP]BA, BA and [9R]BA since these compounds were tentatively identified in morphogenetic tissues of the species tested. LETHAM and PALNI (1983) as well as BAYLEY, VAN STADEN, MALLETT and DREWES (1989) suggested that these compounds are the active forms of the cytokinin. This could well influence organogenesis and even more so when concentrations of these compounds are regulated by auxin concentration.

# CHAPTER 7

# GENERAL CONCLUSION

This study on the effect of benzyladenine on adventitious shoot formation in *Lycopersicon* species was executed in order to determine the reason for the recalcitrance of tomato callus tissue to regenerate *in vitro*. This problem was approached by examining the effects of different growth regulators on adventitious shoot formation, the effects of BA pulses on regeneration of shoots, the uptake and metabolism of BA as well as the influence of auxin on the metabolism of BA in leaf and callus tissue of both *L. esculentum* cv. Rodade and *L. peruvianum*.

Studies on the effects of different growth regulators showed significant differences between the treatments in the two tissue types of both species and also emphasized the importance of the auxin:cytokinin balance with respect to the regeneration of adventitious shoots at an exogenous as well as an endogenous level (THORPE, 1982). It was also shown that BA is superior to zeatin with respect to the regeneration of adventitious shoots under the conditions tested. The tissue types

of both *L. esculentum* and *L. peruvianum* showed a specific IAA concentration above which a decline in shoot production was detected. An optimal concentration was also detected for BA with respect to the induction of adventitious shoots. Tissues of *L. peruvianum* had a much higher morphogenetic potential than comparable tissue of *L. esculentum* (TAL, DEHAN and HEIKIN, 1977). This study has shown that it is possible to regenerate adventitious shoots from callus and leaf tissue of *L. peruvianum* and from leaf tissue of *L. esculentum* cv. Rodade. Callus tissue of *L. esculentum* did however, not regenerate under the conditions tested. It was also evident from this study that other factors are involved during the initiation of adventitious buds in callus tissue of tomato.

In an attempt to further optimize the conditions for regeneration of adventitious shoots in leaf and callus tissue of both species the effects of different incubation periods, solid and liquid media as well as cytokinin pulses were investigated. It was not possible to induce the production of adventitious shoots in callus tissue of the cultivated tomato under the conditions tested and it was only possible to improve regeneration in callus tissue of *L. peruvianum*. Unlike the findings of NORDSTROM and ELIASSON (1986) with apple tissue, a continuous supply of BA was unnecessary for the production of adventitious shoots in both species. A stepwise increase in the percentage shoots produced was observed indicating a

period of induction wherein exposure to BA is beneficial to the production of shoots. Similar results have been reported by DE VILLIERS, JANSE VAN VUUREN, FERREIRA and VAN STADEN (1993).

Different methods of application produced minor improvements in the regeneration of adventitious shoots, unlike results found with mature cotyledons of *Helianthus annuus* (CHRAIBI, CASTELLE, LATCHE, ROUSTAN and FALLOT, 1992) where an improvement in regeneration was noted during exposure to a liquid medium. It was however, shown that leaf tissue is much more responsive to these treatments than callus tissue of both species and that exposure of the tissue to BA is only necessary for a short period in order to induce the production of adventitious shoots *in vitro*.

With respect to the uptake of BA it was found that a linear uptake occurred during the entire incubation period in all the tissue types tested. This finding is in accordance with those of NORDSTROM and ELIASSON (1986). The rate of BA uptake was well correlated with the shooting response found during exposure experiments. As the uptake of BA seems to be limited by the availability of the cytokinin, as well as the rigidity of the gel matrix (BORNMAN and VOGELMANN, 1984), the shooting response is therefore probably also influenced

by these factors.

During studies of the metabolism of BA, results were found that suggest that more similarities are present between the different tissue types of the two species than originally expected. The results also suggest that the main route of BA metabolism is from BA to [9R]BA and that [9R-MP]BA is produced from [9R]BA. A great deal of interconversion was also found between BA, [9R]BA and [9R-MP]BA in both tissue types of the two species. Callus tissue of *L. esculentum* cv. Rodade converted BA to the 3- and 9-glucosides of BA rather than to metabolically active forms such as [9R]BA or [9R-MP]BA (BLAKESLEY, LENTON and HORGAN, 1991). It would further appear that BA was not metabolized to [9R-MP]BA in callus tissue of the cultivated tomato to the same extent as in other tissue types. This may result in endogenous levels of [9R-MP]BA that is much to low to initiate the production of adventitious shoots particularly if this compound constitutes one of the active forms of the cytokinin. The ability to convert the 3-, 7- and 9-glucosides of BA back to active forms of BA seems to be poorly developed in callus tissue of *L. esculentum* cv. Rodade.

During studies on the effect of IAA on the metabolism of BA a system of BA conversion similar to a cytokinin oxidase system was detected in the tissue types.

These results compared favourably to the findings of PALNI, BURCH and HORGAN (1988). It was also shown in this study that IAA plays a definite role in the conversion of BA to [3G]BA and [9G]BA in leaf tissue of *L. esculentum* but that IAA had no apparent effect on the same metabolic process in callus tissue of *L. esculentum*. Results also suggested that the system responsible for the IAA dependant conversion of BA to [9R]BA and [9R-MP]BA in leaf tissue of *L. esculentum* is either inactive or non-existent in callus tissue of cultivar Rodade. In *L. peruvianum* the spectrum of metabolites recovered as well as the trend in the conversion of these metabolites were similar in both leaf and callus tissue. It is therefore clear that IAA interacts with BA in a complex way to control the endogenous levels of BA and its metabolites (HANSEN, MEINS and MILANI, 1985).

The results presented here reflects the complexity of the process of adventitious shoot formation *in vitro*. During these studies, leaf and callus tissue of both *L. esculentum* cv. Rodade and *L. peruvianum* were used in comparative studies. Although the tissue types were different, a large number of similarities exist with respect to regeneration, reaction to BA, uptake of BA, the metabolism of BA as well as the influence of IAA on the metabolism of BA. As in many other tissue types (GEORGE and SHERRINGTON, 1984) the auxin:cytokinin balance (in this

study IAA:BA) is also of major importance with respect to the induction of adventitious shoots in both species under investigation. The wild species has a much higher morphogenetic potential but the same trends in regeneration pattern exist for both species. When the different tissue types were exposed to BA, induction of adventitious shoots occurred within a short period and regeneration could not be improved by either solid or liquid medium. However, the rate of BA uptake was linear in all tissue types and cytokinin pulses had no dramatic effect on regeneration. In all the tissue types, except for callus tissue of *L. esculentum* cv. Rodade, results suggested that the main route of BA metabolism is from BA to [9R]BA and [9R-MP]BA. Callus tissue of tomato on the other hand showed no detectable concentrations of [9R-MP]BA and the storage forms of the cytokinin became very prominent. During the metabolism of BA, IAA played an important role in all tissue types except once again in callus tissue of cultivar Rodade where the auxin had virtually no effect on BA metabolism. The above results therefore suggest that callus tissue of *L. esculentum* cv. Rodade may have inactive or poorly developed systems for the metabolism of benzyladenine *in vitro* and that callus tissue of this important tomato cultivar may for this reason be unable to regenerate adventitious shoots *in vitro*.

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