AFRICAN YAM BEAN: MORPHOLOGY, CLONAL PROPAGATION AND NITROGEN FIXATION

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"Neglected and underutilised species represent a powerful ally in our quest to eliminate hunger and poverty in the next century"

International Plant Genetic Resources Institute, 2001

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ABSTRACT

Morphological and growth observations made on landraces of African yam bean (AYB) used in this study confirm that this species is the most morphologically variable in the genus (Potter, 1992). Morphological characters such as seed colour, stem colour, internode length, leaf size and number of leaves per plant were found to vary between landraces. Growth and development was controlled by both genotype and environment. Flowering was observed only when plants were grown at 25°C, rather than at 30°C, with a 12 h photoperiod. Tuber formation occurred only in AYB5 and not in other landraces. Growth rates differed between landraces and between environments with plants grown in the soil displaying faster growth than those grown in pots. The response to the environment (pot and soil experiment) differed between landraces, i.e. AYB1 performed better than AYB2 in the pot experiment, whereas it was surpassed by AYB2 in the soil experiment.

Clonal propagation protocols were developed using nodal explants/propagules to reproduce material with a high level of genetic uniformity from existing shoot meristems. Clonal propagation was investigated using macro (leafy stem cuttings) and micro (*in vitro* propagation from nodal stem segments) approaches. Axenic shoot cultures have been achieved from stem nodal segments sterilised with 10% "Domestos" bleach and grown in MS-based medium fortified with cytokinins. Amongst the cytokinins used, BAP (6-benzylaminopurine) was found to be more suitable than TDZ (N-phenyl-N′-1,2,3thidiazol-5-ylurea) and 2iP (6-(γ,γ-dimethylallylamino)purine) at both culture establishment and shoot multiplication stages, although optimisation of the protocol for shoot multiplication requires further study. There was persistent callus proliferation at both the establishment/initiation of cultures and the multiplication stage and the use of other plant growth regulators, such as GA_3 (Gibberellic acid) and TIBA (2,3,5-triiodobenzoic acid), known to counter callus growth in cultures, did not give positive results. Although *in vitro* adventitious root formation was erratic, some shoots were able to root when exposed to auxins (IBA [indole-3-butyric acid] and NAA [α-

naphthaleneacetic acid]) and were established in compost. IBA was preferable to NAA, as it induced more root formation. Overall, AYB cuttings produced adventitious roots relatively easily with or without auxins. Auxins at low concentrations induced rapid formation of roots in high numbers. Unlike *in vitro* rooting, adventitious rooting of cuttings was as high as 100% without any auxin treatment, suggesting a possibility of other factors involved in the rooting process *in vitro*.

A cheap source of nitrogen for AYB is in the form of biological nitrogen fixation. AYB nodulated profusely with strains of both a slow growing *Bradyrhizobium* sp. and a fast growing *Rhizobium* sp., plants forming nitrogen fixing nodules with strains ORS302, CP279 and NGR234. Nitrogen fixed from the atmosphere accounted for 79-98% of the plant nitrogen and supported plant growth by an increase of up-to 1547% of dry matter in shoots.

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ABBREVIATIONS AND SYMBOLS

%	percentage
<	smaller than
=	equal
>	greater than
±	plus-minus
≥	greater than or equal to
°C	degree Celsius
µ	micro
µmol	micromole
2,4-D	2,4-dichlorophenoxyacetic
2iP	6-(γ,γ-dimethylallylamino)purine
ADP	adenosine diphosphate
ANOVA	analysis of variance
ARA	acetylene reduction assay
ATP	adenosine triphosphate
AYB	African yam bean
BA	6-benzylaminopurine
BAP	6-benzylaminopurine
BNF	Biological Nitrogen Fixation
C_2H_2	acetylene
C_2H_4	ethylene
CH	casein hydrolysate
cm	centimeter (s)
cm^2	square centimeter (s)
cm^3	cubic centimeter (s)
cpDNA	chloroplast-deoxyribonucleic acid
CRD	complete randomized design
d	day (s)

d.f.	degrees of freedom
DAT	days after transplanting
DMF	*N,N*,-dimethylformamide
F	*F*-table value (treatment *d.f.*, residual/error *d.f.*)
FAO	Food and Agriculture Organisation
g	gramme (s)
GA_3	Gibberellic acid
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour (s)
H^+	hydrogen ion
ha	hectare
HCL	hydrochloric acid
i.d	internal diameter
i.e	that is
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IMS	industrial methylated spirit
K_2HPO_4	potassium phosphate dibasic
K_2NO_3	potassium nitrate
Kg	kilogram (s)
KH_2PO_4	potassium phosphate monobasic
KOH	potassium hydroxide
l	litre (s)
m	metre (s)
M	molar
Mg	magnesium
mg	milligram
$MgSO_4.7H_2O$	magnesium sulfate
min	minute (s)
ml	milliliter (s)

mm	millimeter (s)
mRNA	messenger- ribonucleic acid
N_2	nitrogen gas
NAA	α-naphthaleneacetic acid
NaCL	sodium chloride
NH_3	ammonia
NH_4^+	ammonium ion
NH_4-N	ammonium-nitrogen
NH_4NO_3	ammonium-nitrate
nmol	nanomoles
NO_3^-	nitrate ion
NPA	*N*-(1-naphthyl)phtalamic acid
O_2	oxygen
P	phosphorus
p	probability
PBA	6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine
PGR	plant growth regulator
pH	negative logarithm of hydrogen ion concentration
PP333	paclobutrazol (N-dimethylaminosuccinamic acid)
psi	pounds per square inch
rRNA	ribosomal- ribonucleic acid
s	second (s)
s.e.d	standard error of difference of means
TDZ	N-phenyl-N′-1,2,3thidiazol-5-ylurea
TIBA	2,3,5-triiodobenzoic acid
tRNA	transfer- ribonucleic acid
UK	United Kingdom
v/v	volume to volume
WHO	World Health Organisation
YEM	yeast extract mannitol

CHAPTER 1
INTRODUCTION

1.1 Importance of African yam bean

African yam bean (*Sphenostylis stenocarpa*) is one of Africa's under-utilized plant species with potential to broaden man's food base. It forms small tuberous roots that contain more protein than sweet potatoes, potatoes or cassava roots and above ground produces good yields (2000 kg ha^{-1}) of edible seeds (National Academy of Sciences, 1979). The leaves are also utilized as a spinach/cooked vegetable (Tindall, 1983). Although it is generally considered a minor crop in most areas of its cultivation, farmers in some areas of eastern Nigeria grow it as an important source of income and it is the major legume they produce (Potter, 1992).

African yam bean (AYB) is a highly adaptable crop capable of producing growth even on acid and highly leached sandy soils of humid lowland tropics (Potter, 1992), an attribute common to most under-utilised food plants known to flourish with little inputs in areas too marginal for conventional crops. It has also been shown to form nitrogen-fixing nodules if inoculated with slow growing *Bradyrhizobium* bacteria (Assefa and Kleiner, 1997). This ability to fix atmospheric nitrogen means the plant will not require large amounts of nitrogen fertilizer to meet growth demands, thus making its production affordable to the resource poor farmers living mainly in areas where it grows. Okpara and Omaliko (1995) reported yield increases of yellow yam (*Dioscorea cayensis*) in an intercrop between AYB and attributed that to the latter's ability to fix atmospheric nitrogen. AYB's climbing habit is also utilised as it can form a living fence where it is grown on stakes around fields of cocoyam (Potter, 1992).

Various nutritional studies have revealed the potential of AYB as an alternative food supplement to most diets consumed in the third world that lack some essential nutrients resulting in severe cases of malnutrition. Oshodi *et al.* (1995) recorded comparatively higher values of amino acids (cysteine, lysine, methionine, phenylalanine and pyrone) in AYB flour than the 1985 FAO/WHO amino acid

reference values recommended as the requirement for infants. In the same analysis, it was also found to be a good source of other essential amino acids. Several food products available to the world's poor are lacking in dietary nutrients and crops with such enormous nutritional potential, such as AYB, will assist the fight against malnutrition if their cultivation is developed. In contrast to this potential, some studies conducted on under-utilized legumes in Nigeria have revealed AYB to be one of the legumes with traces of anti-nutritional substances (Oboh *et al.*, 1998). However, some food processing measures, such as dehulling, soaking and soaking/cooking, have been found to reduce significantly the contents of some of these anti-nutritional substances (Nwinuka *et al.*, 1997), thus making AYB more acceptable for human consumption. In other studies, for example, Okeola and Machuka (2001), anti-nutritional substances such as lectin in AYB have been found to possess some insecticidal properties that can be further exploited to benefit food security by reducing storage losses.

1.2 Background on African yam bean

1.2.1 Taxonomy and botanical description

African yam bean, *Sphenostylis stenocarpa,* is one of three taxa used by humans from the large genus *Sphenostylis* E. Meyer (Leguminosae: Papilionoideae: Phaseoleae) comprising 7 species that occur in dry forests and in open or forested savannas in tropical and southern Africa. It is the most widely distributed and morphologically variable species in the genus and by far the most important economically (Potter, 1992).

The plant is a perennial prostrate or climbing herb from a tuberous rootstock with the stem often reddish, glabrous or sparsely puberulus, and woody near the base (Verdcourt and Døygaard, 2001). Roots develop starchy tubers that serve as organs of perennation when the above ground parts die back during the dry season (National Academy of Sciences, 1979; Potter, 1992; Klu *et al.*, 2001). The plants bear leaves that are pinnately trifoliate with linear, lanceolate, ovate or elliptic leaflets and depending on the landrace, they bear from two to twelve purple to magenta coloured flowers with twisted standard petals on pseudoracemes (Potter, 1992). These flowers are produced profusely in 100 to 150 days, yielding slightly woody pods measuring up to 30 cm long and containing from 20 to 30 seeds that reach maturity within 170

days (Klu *et al.*, 2001). The seeds are brown, white, speckled or marbled in colour with a dark brown hilum border and are ellipsoid or round in shape measuring approximately 9×7 mm (Tindall, 1983). A more detailed botanical description of AYB can be found in Potter (1992) and Verdcourt and Døygaard (2001).

1.2.2 Origin of African yam bean

AYB is grown widely in West and Central Africa, but its place of origin within Africa is not known with certainty because its domestication cannot be traced to one locality (Potter and Doyle, 1992). This uncertainty is attributed to the idea that African agriculture is noncentric and thus African crops, such as AYB, are not assignable to a particular site of origin, but should be categorised according to the ecological zones in which they were domesticated. AYB is also believed to have originated from Ethiopia and spread to many areas of tropical Africa where it is found growing wild (Tindall, 1983). A study to try to locate the possible place of origin, though not very conclusive, favoured two independent domestications shown by both cpDNA results and linguistic data (Potter and Doyle, 1992). It concluded that AYB has West and Central African regions as its areas of domestication, where it is grown for seed and tuberous roots, respectively.

1.2.3 Distribution and cultivation of African yam bean

AYB seems little affected by altitude and flourishes at elevations ranging from sea level to 1800 metres (National Academy of Sciences, 1979). It is found growing wild throughout much of tropical Africa and is common in central and western Africa, especially Nigeria. The occurrence of wild races can also be traced to east and southern African regions (Potter and Doyle, 1992). Although collection of the wild plants for consumption seems to be in decline, there is some evidence of the practice especially in the Zaire/Congo area where tubers of a narrow-leaflet landrace are harvested from the wild, cooked and eaten as a vegetable (Potter, 1992).

AYB is widely cultivated in most countries in West Africa, particularly in Guinea, Ivory Coast, Nigeria and Togo extending to parts of central and equatorial Africa (Tindall, 1983). In all areas of its production, it seems to be grown mainly as an intercrop with such crops as maize (*Zea mays* L.) and cassava (*Manihot esculenta* Crantz) where it twines around their stems for support (Potter, 1992; Klu *et al.*,

2001). In an extensive study of *S. stenocarpa*, Potter (1992) made several observations. In major areas of production in Zaire, it is grown as an annual where it is propagated by seed planted in September/October for tubers to be harvested seven to eight months later. Similarly, though for seed consumption, in most parts of Nigeria AYB is grown as an annual, but plants have been seen to regrow from rootstocks after the dry season every year and some plants have been maintained for more than 20 years. Seeds also take the same time as tubers from planting to harvest, normally from April/July to December. In some areas of Ghana, it is also cultivated as a minor crop in mixed cropping systems with major crops, where it benefits from support provided by their stems (Klu *et al.*, 2001). This support is very valuable to the plant since better yields for both seeds and tubers have been obtained when planted with such crops as maize and yams (Potter, 1992). In the mixed cropping system mentioned above, no special attention is given to the crop but it benefits from occasional cultural practices applied to the major crops.

In the same study, Klu *et al.* (2001) revealed a decline in production with limited quantities of the product offered for sale in local markets, even though the price compared favourably with cowpea or groundnut. Improved varieties are not available and crop establishment is usually achieved through the use of landraces that farmers keep from previous harvests or in some cases collect from the markets. Lack of improved varieties with dwarf erect architecture, shorter growth period and easier to cook seed coats have been identified as obstacles to large scale commercial cultivation (Klu *et al.*, 2001).

1.3 Motivation and justification of the present study

The potential of AYB cannot be over emphasised. However, like many other crops of the third world it is still under-utilized because of inadequate information on its physiology, agronomy, lack of good planting material and improved varieties. Due to the restricted attention it receives in terms of production and research, it faces eminent danger of extinction/erosion. It is also loosing out to major legume and tuber crops such as cowpea (*Vigna unguiculata*) and potato (*Solanum tuberosum*) that have been improved for better yield, quality, disease and pest resistance. Like many other under-utilised crops, its survival as a crop has largely been sustained

through tradition and knowledge of the local growers. This is evident from the paucity of documented information on its culture in general.

The study reported here was carried out in order to document culture techniques that can benefit further investigations as well as production of AYB as an alternative food crop. The study is divided into three principal areas viz. clonal propagation protocols, characterisation of plant growth and biological nitrogen fixation.

In this study, clonal propagation protocols have been developed using nodal explants/propagules to reproduce confidently material with a high level of genetic uniformity from existing shoot meristems. Clonal propagation was investigated using macro (leafy stem cuttings) and micro (*in vitro* propagation from nodal stem segments) approaches. Axenic shoot cultures have been achieved from stem nodal segments using the cytokinin BAP in the culture medium and shoots generated have been rooted *in vitro* when exposed to auxins (IBA and NAA) and successfully established in compost. Optimisation of the protocol for shoot multiplication requires further study. Although tissue culture is possible for the mass production of plants, its overall cost limits its application and, therefore, a more affordable alternative technology of propagation from stem cuttings was also investigated. It was established that AYB is very amenable to propagation by cuttings with or without auxins; however, auxins at low concentrations were found to confer benefits of rapid formation of roots and high numbers of roots.

Various disciplines aimed at increasing knowledge of plants are either based on plant morphology or are closely related to it (Claben-Bockhoff, 2001), thus plant morphology, including morphogenesis, remains relevant to practically all disciplines of plant biology (Sattler and Rutishauser, 1997). It is, therefore, important for growth and development of AYB to be quantified, and this was achieved for the two landraces grown in pots and in soil under glasshouse conditions.

Although it makes up 80% of the earth's atmosphere, nitrogen is still the most limiting nutrient for plant growth and the most deficient in soils, and soil deficiencies are recognised to be a major cause of low crop yields in Africa (Dakora and Keya, 1997). Legumes are known to fix atmospheric nitrogen through root nodules

developed in association with some rhizobia and AYB has been reported to form nodules and fix atmospheric nitrogen in association with *Bradyrhizobium* sp. AUEB20 isolated from the Ethiopian tree *Erythrina brucei* (Assefa and Kleiner, 1997). This potential was explored further in this study to establish its promiscuity in nodulation and its efficiency in supporting crop growth from the fixed nitrogen. It was established that AYB forms nodules with a variety of rhizobia and is able to derive adequate nitrogen from the atmosphere to sustain its growth.

CHAPTER 2
LITERATURE REVIEW

2.1 Clonal propagation of plants

Two principles are recognised in the propagation of plants; vegetative (asexual, also called cloning) and generative propagation (sexual, by seeds) (Pierik, 1987). *In vivo* vegetative propagation (cuttings, splitting or division, layering, earthing up, grafting and budding) is often adopted when generative propagation becomes unsatisfactory due to failure of seed set, low seed yield, short seed longevity and heterozygosity of the progeny. If, however, conventional methods of *in vivo* vegetative propagation fail for some reason, *in vitro* vegetative propagation or micropropagation methods are usually adopted.

2.1.1 *In vitro* propagation

The value of tissue culture has long been realised in the field of biotechnology and in plant science research in general. It enables clones to be produced under highly uniform conditions where the environment and nutrient media can be carefully manipulated (Lawrence, 1981).

Tissue culture is made possible by the property of totipotency inherent in living cells described under the cell theory of Schwann and Schleiden (1838-39) that characterises the cell as the smallest biological unit capable of developing into a complete plant (Pierik, 1987). There are several types of *in vitro* culture procedures. Pierik (1987) lists six types, viz. the culture of intact plants (as in seed germination), embryos, organ explants, callus, single cells and protoplasts. George and Sherrington (1984) divide methods of *in vitro* plant propagation into two principal categories, namely shoot multiplication from meristematic tissues (axillary and apical buds) and formation of adventitious shoots (direct organogenesis or indirect organogenesis) on pieces of tissue/organs or unorganised callus tissues. The adventitious formation or *de novo* formation of organs that were not present at the time of isolation of an explant is a very complex process because existing correlations have to be broken down before new ones can be built up which will lead

to organ regeneration (Pierik, 1987). Genetic aberrations that sometimes accompany such approaches make propagation by adventitious organogenesis not suitable for the production of clones. The occurrence of these genetic variations and mutations is the greatest disadvantage of these *in vitro* methods of vegetative propagation (Pierik, 1987). Pellegrineschi (1997) observed somaclonal variation following the *in vitro* regeneration of cowpea via organogenesis. Due to the unreliability of adventitious shoot regeneration as regards perpetuation of a clone, the shoot multiplication by use of organised tissues (existing meristematic tissues) is still the most favoured technique. Isolated buds (axillary or apical) are allowed to develop into shoots, resulting in progeny identical to the mother plant because the process of de-differentiation and re-differentiation of the cellular structures is avoided.

Success of an explant to regenerate complete plants is governed by the interaction between its genotype and environment. An enabling environment is necessary for the realisation of an organism's genetic potential, hence, success of any *in vitro* technique is dependent on the provision of a conducive environment. High tissue growth and regeneration is obtained through the utilisation of well-developed *in vitro* culture techniques/protocols that provide optimal conditions for physiological functions of the explant. Various approaches for optimisation of the necessary conditions have been described and utilised in many different plants (Mercier *et al.*, 1992; Cearley and Bolyard, 1997; Tavares *et al.*, 1998; Girija *et al.*, 1999; Rout *et al.*, 2000; Akasaka *et al.*, 2000; Ibrahim and Debergh, 2001; Kieffer *et al.*, 2001).

2.1.1.1 Plant material/propagule

The genetic makeup and physiology of the plant propagules have far reaching effects on regeneration ability. Different plant genotypes interact differently with the environment and the physiological condition of the material governs the capacity to respond to the environmental stimuli. Pierik (1987) summarised the influence of the plant material as emanating from the genotype, age of the plant and tissue/organ, physiological state, health of the plant, position of the explant within the plant and general growth conditions of the mother/source plant. Thus, selection and management of the source plant is an important aspect of successful micropropagation (Hartmann *et al.*, 1997)

2.1.1.2 Nutritional requirements (macro and micro-elements and sugars)

Optimal nutrition is important for plant growth and development both *in vivo* and *in vitro*. Without nutrients, plant growth and development fail. Different genotypes and explants behave differently to culture conditions and hence, in order to realise the total genetic potential of the cells, it is also important to furnish them with a medium that is fully competent to make them grow (Moore, 1989). In tissue culture, the medium volume per explant is usually relatively small and is not replenished for a long period, therefore, it is pertinent that medium for maximum growth should provide the tissues with sufficient essential nutrients for the duration of the culture so that the depletion of such nutrients does not limit growth (Jeong *et al.*, 1995). Different formulations for media are available and those commonly used are MS medium (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and Woody Plant Medium (McCown and Lloyd, 1981). MS medium is the most popular as most plants react favourably to it, but its content of salt is too high for some plants (Pierik, 1987). The success of plant tissue culture as a means of plant propagation is greatly influenced by the culture medium selected (George and Sherrington, 1984).

2.1.1.3 Plant growth regulators and other organic substances

Plant growth regulators (PGRs) are organic compounds other than nutrients (supplying energy or mineral elements) that, in small amounts, promote, inhibit or otherwise modify any physiological process in plants (Basra, 2000). Plant growth regulation by hormones within the plant is directed by the genetic code within the DNA of the chromosomes and transcription and translation functions of mRNA, tRNA and rRNA during protein synthesis (Hartmann *et al.*, 1997). The PGRs include both naturally occurring plant growth substances or phytohormones and synthetic compounds or chemical analogs. There are five categories of phytohormones; auxins, cytokinins, gibberellins, abscisic acid and ethylene. Several other compounds capable of regulating various facets of plant growth and development have been recently described such as oligosaccharins, brassinosteroids, jasmonates, salicylates and polyamines (Basra, 2000). The success of tissue culture work is determined to a large extent by the levels and kinds of plant growth regulators included in the culture medium (Rout *et al.*, 2000).

Auxins play an important role in the promotion of root growth in many plant species (Nikam and Shitole, 1997), while cytokinins are recognised to play an opposing role to auxins (Moore, 1989). Cytokinins are known to promote growth through their recognised rôle in influencing cell division. Cytokinins used to induce shoot growth frequently inhibit root formation and, usually, such plants do not form roots *in vitro* until they are cultured on a medium with or without auxins. Sometimes, sufficient cytokinin is carried over to inhibit root formation in the rooting medium (George and Sherrington, 1984), so it may be necessary to subculture the plantlets to remove the cytokinin residues. The exogenous regulator requirement (i.e. type of regulator, concentration, auxin/cytokinin ratio) depends strongly on the genotype and endogenous hormone content (Pierik, 1987) because there are explants that produce enough hormones not to warrant addition of any extra regulators in the medium. Gibberellins are also naturally occurring growth substances that induce growth but are not generally known to favour organ initiation (Rout *et al.*, 2000) and usually inhibit adventitious root formation (George and Sherrington, 1984; Pierik, 1987).

Other plant growth regulators that have been observed to play a rôle in *in vitro* plant regeneration are auxin polar transport inhibitors and gibberellin biosynthesis inhibitors. Auxin polar transport inhibitors are usually used to restore regeneration capacity of some explants. It has been suggested that addition of such compounds to regeneration media might inhibit the transport of endogenous IAA to regeneration sites, so that an auxin/cytokinin balance becomes more favourable for the regeneration of shoot buds (Nakano *et al.*, 2000). Since gibberellins are well known to promote growth but do not favour organ initiation such as adventitious root formation, addition to the culture medium of certain chemicals that block biosysnthesis of gibberellins has been reported to give positive results (McKinless and Alderson, 1993).

2.1.2 *In vitro* regeneration of legumes

Most legume plants are propagated easily generatively *in vivo*. However, due to shortage or unavailability of good seed, it is sometimes difficult to obtain enough plants or the progeny may be highly heterogenous. Therefore, *in vitro* propagation may be used to produce many plants rapidly to generate clones. Although an excellent method of propagation, *in vitro* culture is not always easily achieved in all

plant families including the Papilionaceae, because some genotypes have less ability to regenerate. Studies with other grain legumes; i.e. black gram (*Vigna mungo*) (Geetha *et al.*, 1997a,b), soybean (*Glycine max*) (Santos *et al.*, 1997; Samoylov *et al.*, 1998), cowpea (*Vigna unguiculata*) (Brar *et al.*, 1999), peanut (*Arachis hypogea*) (Chengalrayan *et al.*, 1998; Ponsamuel *et al.*, 1998), pigeonpea (*Cajanus cajan*) (Geetha *et al.*, 1998; Screenivasu *et al.*, 1998; Singh *et al.*, 2003), rice bean (*Vigna umbellata*) (Bhadra *et al.*, 1991), adzuki bean (*Vigna angularis*) (Chitra and Padmaja, 2002), mung bean (*Vigna radiata*) (Avenido and Hattori, 2001; Devi *et al.*, 2004), common bean (*Phaseolus vulgaris*) (Zambre *et al.*, 1998) and tepary bean (*P. acutifolius*) (Zambre *et al.*, 1998), have demonstrated the effectiveness of *in vitro* techniques as propagation tools thus offering hope for application in the propagation of AYB.

2.1.2.1 *In vitro* shoot growth and multiplication from nodal explants

As previously mentioned, due to unreliability of adventitious shoot regeneration as regards perpetuation of a clone, shoot multiplication by use of organised tissues (existing meristematic tissues) is still the most favoured technique because the resulting progeny is identical to the mother plant. Various factors and their interaction have been observed to play an important role in the success of *in vitro* propagation of many plants. Stimulation of *in vitro* growth from meristematic explants has been recorded by many researchers (Kallak and Koiveer, 1990; Fenning *et al.*, 1993; Bennet *et al.*, 1994; Yang and Read, 1996; Ramanayake and Yakadawala, 1997; Luo and Jia, 1998; Tavares *et al.*, 1998; Chitra and Padmaja, 1999; Girija *et al.*, 1999; Naik *et al.*, 1999; Akasaka *et al.*, 2000; Bag *et al.*, 2000; Bordon *et al.*, 2000; Ebrahim and Ibrahim, 2000; Mhatre *et al.*, 2000; Naik *et al.*, 2000; Dielen *et al.*, 2001; Ibrahim and Debergh, 2001; Ramirez-Malagon *et al.*, 2001; Lu, 2002; Mereti *et al.*, 2002; Chaturvedi *et al.*, 2004).

Various natural and synthetic cytokinins are used to stimulate growth and development in tissue culture, kinetin, 6-benzylaminpurine (BAP), 6-(γ,γ-dimethylallylamino)purine (2iP) and 6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine (PBA) being in common use (Pierik, 1987). The effects of cytokinins were found to be dependent on many factors, including concentration and type of cytokinin, genotype, type of explant and other growth regulators such as auxins.

Amongst the cytokinins used in tissue culture, BAP produced the highest efficacy in shoot/bud proliferation (Yang and Read, 1996; Brar *et al.*, 1997). Increased concentration of BAP from 1.0 to 5.0 mg l^{-1} resulted in increased shoot bud production of black gram (*Vigna mungo*) (Geetha *et al.*, 1997b). In the same study, a high frequency of shoot-bud differentiation was observed at 3.0 mg l^{-1}. In some instances, high concentrations were observed to favour callus proliferation and inhibited shoot growth. High concentrations of BA caused browning of cultures, less vigorous thin shoots and shoot tip decay of chickpea (*Cicer arietinum*) (Polisetty *et al.*, 1997).

Incorrect media formulations have been blamed, in some instances, for failure of regeneration, or the abnormal growth and development of some explants (Akasaka *et al.*, 2000). High concentration of BA stimulated shoot bud formation but adversely affected the rate of shoot differentiation from seed explants of chickpea (*Cicer arietinum*) (Polisetty *et al.*, 1997). Avenido and Hattori (2000) recorded significant reduction in adventitious regeneration of shoots and increased callusing from hypocotyls of adzuki bean (*Vigna angularis*) when the concentration of BA was increased twofold. A high frequency of shoot-bud differentiation was achieved on MS medium supplemented with BAP (3.0 mg l^{-1}) and NAA (1.0 mg l^{-1}), and about 90% of shoot tips produced up to 15 shoots per culture in the case of Black gram (*Vigna mungo*) (Geetha *et al.*, 1997b). Approximately 80% of the shoots with roots survived in the field and produced phenotypically normal plants thus ensuring perpetuation of clonal material *in vitro*. Similar trends were observed in the induction of shoot tip multiplication of cowpea from shoot tip explants (Brar *et al*, 1997) and, in chickpea (*Cicer arietinum*), complete plant regeneration has been reported from seed explants (Polisetty *et al.*, 1997).

Sufficient nutrient salts are essential to avoid limited culture growth due to nutrient stress. Most formulations referred to here used MS salts as basal medium because most plants respond favourably to it (Pierik, 1987). Since it is not optimal for all plant species, adjustments are sometimes imposed or an alternative formulation used. MS medium adjusted to half of the original strength produced both callus and shoot buds while full strength MS inhibited bud formation of black gram (*Vigna mungo*) (Das *et al.*, 1998). Ohki and Sawaki (1999) established the benefit to shoot

proliferation of *Delphinium cardinale* by adjusting the strength of MS medium by one third. Chaturvedi *et al.* (2004) reported that half strength MS medium required initially to establish nodal segment cultures of neem tree (*Azadirachta indica*) also stimulated subsequent multiple shoot formation when enriched with BAP (1μM) + GA_3 (0.5 μM) or casein hydrolysate (CH) (250 mg l^{-1}). However, these media did not support shoot growth and the shoots remained compact and stunted until MS was used at full strength. An alternative to the adjustment of MS medium is to use other salt formulations such as B5 and WPM. MS medium was effective for induction and initiation phases of *Fraxinus angustifolia* but replaced by DKW medium (Driver and Kuniyuki, 1984) for bud development (Tonon *et al.*, 2001). Carelli and Echeverrigaray (2002) achieved an improved explant survival of rose (*Rosa hybrida*) on QL medium (Quoirin and Lepoivre, 1977) (medium with relatively low ammonium ion concentration, increased calcium and chlorine ions to nearly zero) than MS and B5 due to low oxidation and probably reduced hyperhydricity. Saadat and Hennerty (2002) also found DKW medium to be optimal for *in vitro* multiplication of Persian walnut (*Juglans regia*) compared to MS and WPM media.

It has been suggested that addition of auxin polar transport inhibitors, 2,3,5-triiodobenzoic acid (TIBA) and *N*-(1-naphthyl)phtalamic acid (NPA), to regeneration media may inhibit the transport of endogenous IAA to regeneration sites, so that an auxin/cytokinin balance becomes more favourable for the regeneration of shoot buds (Charriere and Hahne, 1998; Nakano *et al.*, 2000). Combination of TIBA and cytokinin (BA or TDZ) restored regeneration potential of 75-month-old cultures of cell clumps of *Lilium formosanum* by a 10-fold increase in the number of regenerated shoot buds but no somatic embryos (Nakano *et al.*, 2000). Addition of auxin transport inhibitors (TIBA and NPA) to medium known to favour somatic embryogenesis of sunflower led to shoot formation instead (Charriere and Hahne, 1998). These observation have shown somatic embryos are replaced by shoots as auxin polar transport inhibitors stimulate direct shoot regeneration from callus without going through embryogenesis. While both observations indicated that the morphogenetic response was found to be dose dependent increasing with concentration, relatively high concentrations were deleterious. Although auxin polar transport inhibitors have been shown to stimulate morphogenesis, this response might vary with species. Shoot tip removal and additional defoliation improved

axillary branching of *Codiaeum variegatum* Blume *var. pictum* Muell. Arg. but addition of TIBA did not replace defoliation nor diminish the size of callus formed at the base of shoots (Orlikowska *et al.*, 2000). In contrast, Voyiatzi *et al.* (1995) demonstrated the benefit of TIBA where, together with other growth inhibitors, it replaced the need for conventional manual shoot tip pinching usually employed to eliminate apical dominance and encourage axillary branching.

Many researchers have recorded a synergistic effect of BAP and GA_3 on *in vitro* shoot growth from nodal explants (Pattnaik and Chand, 1997; Purohit and Singhvi, 1998; Chitra and Padmaja 1999; Vengadesan *et al.*, 2002). Addition of GA_3 to medium with an elevated BAP concentration facilitated the elongation of *in vitro* grown shoots of mulberry (*Morus indica* L.) that was otherwise impaired (Chitra and Padmaja, 1999). They observed no enhancement of multiple shoot induction when the concentration of BAP was increased above 1.5 mg l^{-1} but shoot tips cultured on medium containing both BAP (4.0 mg l^{-1}) and GA_3 (0.05 mg l^{-1}) resulted in shoot elongation followed by sprouting of axillary buds which also developed into shoots. Pattnaik and Chand (1997) achieved faster bud break both in apical shoots and nodal explants and an enhanced frequency of bud break in three mulberries species through the incorporation of GA_3 (0.2-0.4 mg l^{-1}) along with BAP (1.0 mg l^{-1}). Nodal segments excised from shoots raised *in vitro* and cultured in medium with 0.4 mg l^{-1} GA_3 and 1.0 mg l^{-1} BAP gave a high number of shoots that was shown with repeated subculture of nodal segments of newly formed shoots. Purohit and Singhvi (1998) reported improved shoot elongation and an enhanced rate of shoot multiplication of *Achras sapota* (L.) when GA_3 (1 mg l^{-1}) was incorporated in the medium during the first subculture after establishment. A single use of GA_3 during subculture eliminated the need for prolonged culturing on BAP. Subculturing onto medium containing GA_3 of stunted shoots that had been proliferated on a medium containing TDZ resulted in considerable elongation of the shoots (Vengadesan *et al.*, 2002).

2.1.2.2 *In vitro* adventitious root formation and growth

Rooting of *in vitro*-derived plants is an important step in micropropagation as the ultimate goal is to have the plants grow *in vivo*. Although some species easily form adventitious roots on shoots produced *in vitro*, other species may require specific treatments before they can grow roots hence the third stage of micropropagation

(George and Sherrington, 1984). Auxins are the most commonly applied PGRs to stimulate rooting and their potency varies with type of auxin, concentration and plant genotype (Pierik, 1987). The naturally occurring auxin IAA is usually used at relatively high concentrations while the synthetic and relatively more active auxins (IBA, NAA or 2,4-D) are used at lower concentrations. Nikam and Shitole (1997) achieved root formation on shoots of niger (*Guizotia abyssinica*) grown on hormone-free media as well as media with hormones. They found no significant difference in the numbers of shoots with roots on media with varying concentrations of IAA and NAA, however, the best root growth in all shoots was in the presence of 0.5 mg l^{-1} NAA whereas higher concentrations of IAA and NAA induced callus at the base of shoots. It is common that low concentrations of auxins lead to adventitious root formation whereas with high auxin concentrations root formation fails and callus proliferation predominates (Pierik, 1987). Girija *et al.* (1999) recorded the best rooting with IBA at all concentrations tested (0.5-2.5 mg l^{-1}), compared to IAA and NAA which promoted callus at the basal cut end of firecracker plant/flower (*Crossandra infundibuliformis*).

Avenido and Hattori (2000) found that explants of adzuki bean grown in the absence of BA exhibited high frequency root formation. They showed that while BA promoted adventitious shoot induction and regeneration it nevertheless effectively inhibited root formation. Polisetty *et al.* (1997) also found that shoots produced under various treatments did not produce any roots upon prolonged culture on BA medium. An auxin requirement in rooting was demonstrated for black gram (*Vigna mungo*) when shoots that failed to root on media devoid of auxin for 15 days rooted within 15-20 d of culture on medium containing different concentrations of IBA (0.1-5.0 mg l^{-1}) (Geetha *et al.*, 1997b). Maximum percentage rooting was observed with 3.0 mg l^{-1} IBA. These observations demonstrate the necessity of different media for shoot growth and rooting for different plant species.

Rooting of species previously known to be slow to root has been achieved with shoots exposed to gibberellin biosynthesis inhibitors. Such chemicals have the capability to block endogenous gibberellin biosynthesis in cultures and thus promote root growth. Several studies have demonstrated the beneficial effect of growth retardants and auxin in root formation (Bora *et al.*, 1991; Sebanek *et al.*, 1991; Nagy

et al., 1991; Sharma and Webster, 1992; Mckinless and Alderson, 1993; Eliasson *et al.,* 1994; Leshem *et al.,* 1994; Wiesman and Lavee, 1994; Wiesman and Riov, 1994; Wiesman and Lavee, 1995; Porlingis and KoukourikouPetridou, 1996; KoukourikouPetridou and Porlingis, 1997; Pan and Gui, 1997; Pan and Tian, 1999; Lakshmanan *et al.,* 2002; Wiesman *et al.,* 2002; Keeley *et al.,* 2003). Growth retardants act in synergy with auxin by interfering with the synthesis of gibberellins, but sometimes they may be acting individually by increasing the level of endogenous IAA such as in *Ligustrum vulgare* cuttings (Sebanek *et al.,* 1991). Rooting was achieved on rhizome buds of *Lapageria rosea* that were proliferated in the presence of the gibberellin biosynthesis inhibitor paclobutrazol (McKinless and Alderson, 1993). As a growth retardant, paclobutrazol may, however, also have deleterious effects on plant growth as observed in delayed seedling emergence and retarded vegetative growth of maize (*Zea mays*) (Khalil and Rahman, 1995) and in inhibition of rooting in bean (*Phaseolus vulgaris*) (Tari and Nagy, 1996).

2.1.2.3 Acclimation and field establishment of *in vitro*-derived plants

Transfer of rooted plants to soil should be done carefully to reduce losses because of the differences between the environment inside and outside tissue culture vessels. Plants growing *in vitro* are heterotrophic because of the media they grow in and, therefore, need to become autotrophic when transferred to the external environment. Leaves developed *in vitro* are so modified anatomically such that stresses of terrestrial environment make them functionally inadequate when such plantlets are transplanted immediately to the glasshouse (Grout and Aston, 1978a). It is thus important for them to adjust gradually from the heterotrophic to the autotrophic state. Two distinct groups of micropropagated plantlets are recognised; those with photosynthetically incompetent *in vitro* formed leaves and those able to adapt to autotrophic conditions (Van Huylenbroeck *et al.*, 1998). In addition to the nutrition, *in vitro* grown plants lack wax on the cuticle necessary to reduce transpirational losses. To ensure survival, it is imperative for *in vitro* plants to have enough starch reserves and also be in a high relative humidity environment to see them through their first days *in vivo*. Alternatively, modification of the *in vitro* conditions to induce full autotrophic capability of *in vitro* derived leaves or attempt to increase the rate of new leaf production after transplanting (Grout and Aston, 1978b; Grout and Millam, 1985).

2.1.3 Propagation of plants by stem cuttings

Although one of the best propagation tools, tissue culture may not be appropriate for many nurseries where facilities or technical expertise to conduct an effective micro-propagation system are absent (Almehdi *et al.*, 2002). Rooting of cuttings of easy to root species is usually used as a method of asexual propagation to achieve genetically identical plants. For the induction of a vigorous root system, a simple and rapid technique is required which will shorten the growth cycle and provide high propagule survival giving rise to vigorous plants (Vesperinas, 1998).

Two types of adventitious roots are recognised: preformed or latent root initials and wound-induced roots (Hartmann *et al.*, 1997). Preformed roots exist naturally on stems and may emerge while stem is still attached to the mother plant or lie dormant until stems are made into cuttings and placed under conditions conducive to root emergence. Wound-induced roots are considered to form *de novo* and develop only after a wound has been made. Formation of adventitious roots on stem cuttings is influenced by a number of factors including hormonal balance (auxins and other plant growth regulators), condition of propagules (physiological status and genotype) and the interaction effects of these factors. Day and Loveys (1998) observed that the response of woody plant cuttings might be species specific and that seasonal variation in the success of propagation may be mediated through changes in the concentrations of endogenous plant growth regulators or carbohydrates. Although the application of auxin (IBA) to the base of cuttings improved the rooting potential of *Cotinus coggygria* 'Royal Purple', negative effects associated with removing a proportion of mature leaves appeared to relate to alteration in the carbon balance rather than an influence on the supply of endogenous auxin to the potential rooting zone (Cameron *et al.*, 2001).

2.1.3.1 Hormonal control of stem rooting

Auxins play an important role in the promotion of rooting in many plant species (Henry *et al.*, 1992; Chee, 1995; Goh *et al.*, 1995; Garrido *et al.*, 1996; Cameron *et al.*, 2001), although they can also be inhibitory to plant growth in general if applied in high concentrations. This observation has been confirmed through several studies where exogenous application of auxin was found to hasten the rate of rooting,

increased root number and final rooting percentage of most species, although in some cases relatively high concentrations resulted in inhibited root formation and growth, reduced bud/shoot growth and even mortalities (Badji *et al.,* 1991; Demeke *et al.,* 1992; Edson *et al.,* 1994; Ofori *et al.,* 1996; Shiembo *et al.,* 1996; Mesen *et al.,* 1997; De Andres *et al.,* 1999; Copes and Mandel, 2000; Fett-Neto *et al.,* 2001; Aminah, 2003; Ercisli *et al.,* 2003). Aminah *et al.* (1995) observed that auxin (IBA) significantly increased the rate of root emergence in leafy stem cuttings of *Shorea leprosula,* but higher doses (more than 20 μg per cutting) reduced rooting. Dunn *et al.* (1996) also found differences in root formation and growth between cuttings of *Pistacia chinensis* treated with different concentrations of IBA. These observations suggest an existence of optimal auxin concentrations for various species, with some species requiring it for rapid root development or for stimulating more roots.

2.1.3.2 Physiological status of propagule/propagation material

Plant tissues need to be supplied with carbohydrates before they will produce adventitious roots and in green tissues this is achieved through photosynthesis (George and Sherrington, 1984). The presence of leaves on cuttings has also been found to be beneficial due to their ability to produce endogenous auxins (Hartmann *et al.,* 1997) as well as assuring necessary metabolic functions (Badji *et al.,* 1991). Several researchers have observed the association of higher rooting percentage and root number with relatively large leaf area (Ofori *et al.,* 1996; Dick *et al.,* 1998; Cameron *et al.,* 2001; Tchoundjeu *et al.,* 2004). Nketiah *et al.* (1998) recorded higher rooting percentage of *Triplochiton scleroxylon* from cuttings with larger leaf area of 100 cm^2 than those with 30, 50 and 80 cm^2. In contrast, (Aminah *et al.,* 1997) observed highest rooting of stem cuttings with small leaf area (15 cm^2) and decreased rooting with increasing leaf area (30 and 60 cm^2) in *Shorea leprosula*, and this was attributed to the greater water loss from the larger leaf area.

2.2 Nodulation and nitrogen fixation

Nitrogen (N) is the most limiting nutrient for plant growth and the most deficient in soils although it makes up about 80% of the earth's atmospheric air. It is an essential plant nutrient and the major constituent of both structural and non-structural compounds in plants. Nitrogen also comprises 16% of proteins and, thus, neither

man, other animals nor plants can survive unless plant roots extract it from the soil (Frink *et al.*, 1999). Thus, its deficiency in the soil often results in reduced crop yields. To redress the problem of deficiency, nutrients are usually imported in the form of mineral fertilizers, but for a variety of social, economic and political reasons this is generally difficult, especially in Africa (Giller, 2001). Where fertilizer is less costly, as in the developed world, it is feared that humanity's increase in the fixation and mobilisation of N by fertilization and combustion is increasing emissions and thus deposition of nitrogen oxides and NH_4-N that can acidify soils and eutrophy waters (Frink *et al.*, 1999). It is also feared that increased depositions of N will lead to loss of genetic diversity where plants favouring high N supply will displace others. An essential element of agricultural sustainability is the effective management of N in the environment (Graham and Vance, 2000) and, in order to combat pollution and sustain aquatic culture/agriculture systems, Gard *et al.* (2001) recommended a reduction in the excessive use of organic and inorganic fertilizers. This usually involves some use of biologically fixed N_2 because N from this source is used directly by the host plant and, thus is less susceptible to volatilisation, denitrification and leaching.

The capability of biological fixation of atmospheric nitrogen (diazotrophy) is restricted to organisms with prokaryotic cell structure, viz. bacteria and blue-green algae (Cyanobacteria) (Marschner, 1995). Three major strategies of N_2 fixation can be differentiated in terrestrial ecosystems, i.e. symbiotic, associative and free-living nitrogen fixing organisms, differing in both energy source and fixation capability (Marschner, 1995). The symbiotic system has the highest fixation efficiency because the nitrogen fixed is rapidly translocated from the organism into the plant. The process is a highly mutualistic relationship between the two organisms because each derives benefit; the host plant (macrosymbiont) provides the bacteria (microsymbiont) with energy material (from the photosynthesis process) while the bacteria converts atmospheric nitrogen (gaseous N_2) to a plant usable form (NH_4^+).

Legumes are known for their ability to fix atmospheric nitrogen through root nodules developed in symbiotic relationship with some strains of gram negative bacteria of the *Rhizobiaceae* (often collectively referred to as rhizobia). There are six genera recognised for root-nodule bacteria of legumes (*Azorhizobium, Bradyrhizobium,*

Mesorhizobium, Rhizobium, Allorhizobium and *Sinorhizobium*). Amongst the six genera, *Rhizobium* (fast growing) and *Bradyrhizobium* (slow growing) are the principal soil inhabitants (Castro *et al.*, 1999) and thus form a nitrogen-fixing symbiosis with most members of the family Leguminosae. These two genera are generally referred to as promiscuous due to a broad host range nodulated by most of their strains. There must be compatibility between the common core elements of the legume symbiosis and the most important general principle is the specificity of the symbiotic relationship established between the rhizobia and their host plants (Giller, 2001).

2.2.1 Nodulation in legumes

The first step in establishment of rhizobial-legume symbiosis is an interaction between a legume species that is susceptible to nodulation and compatible rhizobia (Giller, 2001). Certain strains of rhizobia may be termed compatible if they form a symbiosis with some legume species or incompatible if they are not able to form a symbiosis with the target host plant. Each rhizobial strain has an established host range, with which it is capable of forming a symbiotic relationship but not with others. Such rhizobia may have been introduced deliberately into the soil by inoculation, or they may already be present in the soil as free living bacteria, in which case they are termed 'indigenous rhizobia'. The rhizobia must first infect or enter the plant and induce root/and or stem nodules to prove the ability or 'infectiveness' of its strain. Infection is achieved through several mechanisms, viz. root hair (deformation) penetration and infection thread formation, direct entry via wounds or sites of lateral root emergence and penetration of root primordia found on the stems of plants (Giller, 2001). The latter mechanisms occur without any infection thread formation and thus are termed direct entry. The modes of penetration differ between legume species. Harrier *et al.* (2000) collectively term the early events in the symbiotic process leading to legume nodulation as 'pre-infection'; they take place in the rhizosphere and involve rhizobial chemostasis/electrotaxis towards the root, root colonization by rhizobia, attachment of rhizobia to surfaces, particularly to emerging root hairs and, in many species root hair deformation and curling. Therefore, effective formation of root nodules requires an introduction of the bacteria as early as possible in the plant establishment. A major component of this initial interaction consists of stimulation of biochemical activity in the rhizobial

strains by flavonoid and isoflavonoid molecules in the plant exudates recognised to have some specific preference on *nod*gene (nodulation gene) activation (Giller, 2001). Flavonoid or isoflavonoid compounds enter the bacterial cell where they bind to a protein termed NodD, ultimately converting it into a transcriptional activator for stimulation of other remaining nodulation genes to synthesize a signal molecule (Nod factor) that is secreted into the rhizosphere.

Infection begins with attachment to immature emerging root hairs and consequent deformation, curling of the root hairs and hydrolysis of root surfaces at the point of infection (centre of the crook) that allows penetration of the infection thread. In response to this supposed invasion, there is evidence of host-cell proliferation in the root cortex adjacent to infected root hairs that become nodule primordia/meristems and consequently rhizobia are released into it. The continued infection thread penetration of the root cortex, host cell division and enlargement results in formation of a visible nodule that can differ in appearance and structure depending on the legume host species. Two types of nodules are recognised; the determinate type, such as those generally found in peanut, and the indeterminate ones similar to those formed on pea and alfalfa (Akasaka *et al.*, 2000). Determinate nodules are round and have no pronounced meristematic region, as compared with the elongated and pronounced meristematic structure of indeterminate ones (Giller, 2001). Many features of the symbiosis are host controlled as a single rhizobial strain is often capable of infecting different host plants by different means, and giving rise to nodules of different structures.

Nodulation alone does not necessarily mean nitrogen fixation. Thus, a further distinction of nodules is brought about by their capacity/ability to fix N_2 from soil air. Nitrogen fixing nodules are characterised by a large pink or red region due to the presence of leghemoglobin protein while the non-active ones are either white or greenish brown. Leghemoglobin (red in colour due to the heme group as in blood hemoglobin) is thought to help transport oxygen (O_2) into the bacteriods at carefully controlled rates because too much of it inactivates the enzyme that catalyses nitrogen fixation (Salisbury and Ross, 1992).

2.2.2 The nitrogen fixation process

Plants use nitrogen only in the combined forms of ammonium (NH_4^+) and nitrate (NO_3^-) ions, but the greatest concentration of N is in the form of dinitrogen gas (N_2) that is not usable by plants. The process of reducing nitrogen to a more available form for plants is very costly in terms of energy which explains why N_2-fixation capability is not universal (Giller, 2001). The reduction of N_2 to the useful form of ammonia is illustrated by the following equation;

$$N{\equiv}N + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_I$$

In biological nitrogen fixation the process is driven by the enzyme nitrogenase found in bacteria and, because plants do not possess it, they are unable to carry out this reaction on their own, but acquire the capacity once in association with the correct rhizobia bacteria through root nodules. The legume as a host supplies the required energy in the form of carbohydrates for ATP synthesis and reducing equivalents (electrons). Nitrogenase enzyme catalyses N_2 and other substrates with triple bonds such as H^+ and C_2H_2 (acetylene); this utilization of other substrates offers an important tool for studies on its activity and N_2 fixation (Marschner, 1995).

The ammonia generated by nitrogenase is assimilated as ammoniun (NH_4^+) ions into glutamine and glutamate (Giller, 2001). This process happens via the joint action of the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), (Marschner, 1995; Giller, 2001). The final fate of the fixed nitrogen is its participation in plant growth as a major building block of proteins; therefore it must be transported to sites of utilization. According to Giller (2001), in legumes the fixed nitrogen is further assimilated and transported predominantly as the amino acids asparagine and glutamine in amine exporters, or as the ureides allantoin and allantoic acid in ureide exporters. Hence there are two types of nitrogen fixing legumes, the amine exporters and ureides exporters based on the composition of their xylem sap. The activity of the enzyme (nitrogenase) involved in the nitrogen fixation reaction as well as the presence of the fixed nitrogen transportation compounds accorded studies in biological nitrogen fixation tools to determine the efficacy or effectiveness of the symbiosis process.

2.2.3 Nodulation and nitrogen fixation in cropping systems

While nitrogen fixing legumes benefit directly from fixed nitrogen, beneficial effects of legumes on succeeding crops arise through a variety of ways (Giller, 2001). Although constrained by various environmental and nutritional factors, including cropping patterns used, in Africa, grain legumes still fix 15-210 kg N ha^{-1} seasonally, thus making them an important component of traditional cropping systems (Dakora and Keya, 1997). One of the important contributions of AYB and other legumes in mixed cropping systems is the contribution to soil productivity. Incorporation of rapidly growing legumes that have high nitrogen fixing capacity plays an important role in crop productivity through improved soil health and fertility (Pretty *et al.*, 2003). The use of AYB and other legumes as cover crops has been shown to increase efficiency of fertilizer utilization and the amount of organic matter for maintenance of high soil productivity (Obiagwu, 1995b). The contribution of nodulating legumes to soil and crop productivity has been widely documented (Dakora and Keya, 1997; Fettel *et al.*, 1997; Rodriguez-Navarro *et al.*, 1999; Graham and Vance, 2000; Hungaria and Vargas, 2000; Mpepereki *et al.*, 2000; Panzieri *et al.*, 2000; Giller, 2001; Maingi *et al.*, 2001; Molla *et al.*, 2001; Zahran, 2001; Fening and Danso, 2002; Aranibar *et al.*, 2003).

The significance of the contribution of nodulating legumes to soil and crop productivity was found to be dependent on the rhizobial strain used and also on the legume/rhizobial strain combination. In beans (*Phaseolus vulgaris*), Rodriguez-Navarro *et al.* (1999) observed that, although there were differences between rhizobial strains in terms of biomass and N concentration in shoots resulting from differences in nitrogen fixation rates, plant genotype significantly modified the performance of rhizobial strains. Inoculation of legumes with an effective strain can significantly replace chemical fertilizer for the supply of nitrogen. Maingi *et al.* (2001) recorded the highest seed dry weights and subsequently yields per hectare of common bean (*Phaseolus vulgaris* L.) from inoculated and N application treatments.

While the legume–rhizobial symbiosis is understood for many major legume crop species, it has been studied less for underutilised crops such as AYB. An endosymbiont (characterized and designed *Bradyrhizobium* sp. AUEB20) isolated from the Ethiopian tree *Erythrina brucei* formed a small number of large,

indeterminate N_2-fixing nodules with AYB (Assefa and Kleiner, 1997). Obiagwu (1995a) recorded the formation of nodules on AYB plants that did not receive any fertilizer and those that received phosphorus, but none in plants receiving nitrogen treatment. In an intercropping experiment where no fertilizer was applied, AYB was one of the crops found to contribute substantially to maize, yam and cassava productivity (Obiagwu, 1995b). Contribution of these legumes to soil productivity was attributed to nitrogen fixation and also to their low nitrogen harvest index that leaves substantial quantities of N behind in their residues and hence available to other crops. In a screening experiment for a suitable cover crop, (Obiagwu, 1997) observed that nitrogen fixation ability of AYB was low in phosphorus deficient soils but improved dramatically under phosphorus application. Tissue nitrogen content increased dramatically from 26 to 203 mg kg^{-1} in no phosphorus treatment to the phosphorus treatment respectively. In the above observations, AYB contributed to nitrogen fixation without any inoculation, thus suggesting that AYB is promiscuous with a wide range of strains.

2.3 Conclusion and hypotheses

Previous work performed in legumes and other plants offer an opportunity of application in the study of AYB.

The main objectives of the study were:

i. Develop clonal propagation protocols that can be used as tools for further studies requiring either genetically uniform material or planting material in place of scarce seed material by testing the hypothesis that AYB is amenable to clonal propagation through the use of stem cuttings (macro propagules) and *in vitro* techniques (micro propagules).

ii. Characterise growth of the plants and variation between landraces through the hypothesis that AYB landraces will respond differently to environment in regard to growth and development [vegetative growth/dry matter accumulation rate and reproductive growth (flowering and seed yield)].

iii. Explore the biological nitrogen fixation capacity of AYB plants and how it can enhance the potential of the species as an alternative food crop for resource poor farmers. The hypothesis is that, since most tropical

legumes are promiscuous with various rhizobial strains, AYB plants will be nodulated by different strains and derive adequate nitrogen from the atmosphere to support growth; rhizobial strains also vary in their infectivity and efficiency to fix atmospheric nitrogen in association with different AYB landraces.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Introduction

Experiments described in this thesis were conducted at the University of Nottingham, School of Biosciences, Sutton Bonington Campus, UK from September 2001 to September 2004. This chapter describes materials and methods common to the whole study and any specific methodologies are described under the relevant chapters.

3.2 Source of plant material

Seeds of five landraces of African yam bean (AYB) were obtained from Professor Richard Mithen of the University of Nottingham, Sutton Bonington, UK with the following information (Table 3.1). The description of the seeds is shown in Plate 3.1.

Table 3.1 AYB landraces used in the study

Landrace	Collector	Area of origion/collection	Part used
DP 860108-01 (AYB1)	Dr. D. Potter University of California Davis	Ohuno, Kwara State, Nigeria	Seed
DP 860117-01 (AYB2)	Same as above	Arugu, Kwara State, Nigeria	Seed
DP 880614-05 (AYB3)	Same as above	Yungu, Bas-Zaire, Zaire/DRC	Root tuber
DP 880614-06 (AYB4)	Same as above	Mbemba Marendi, Bas-Zaire, Zaire/DRC	Root tuber
DP 860108-02 (AYB5)	Same as above	Alife-Kese, Bendel State, Nigeria	Seed

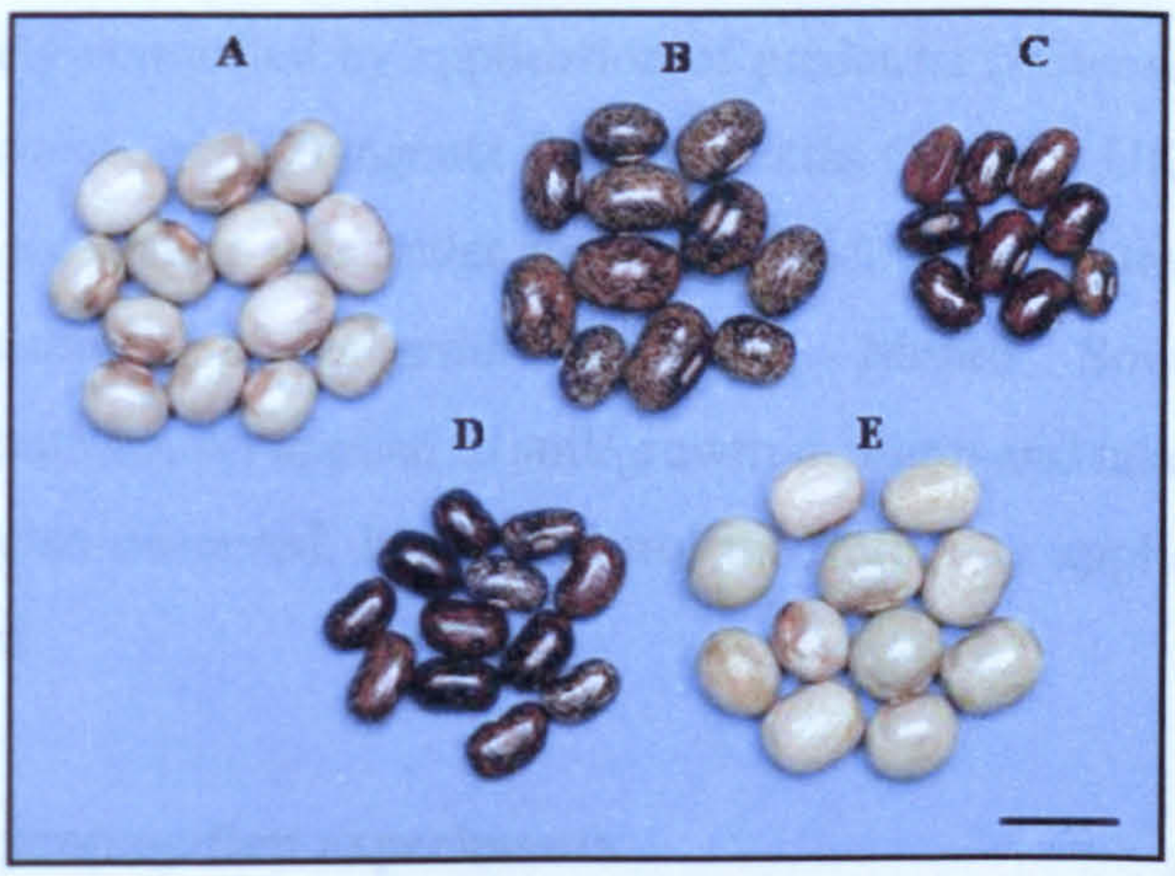

Plate 3.1 Colour, shape and size variation of seeds of AYB landraces [(A) AYB1, (B) AYB2, (C) AYB3, (D) AYB4 and (E) AYB5] used in the study. Bar = 10 mm.

3.3 Establishment and maintenance of plants

Seeds were surfaced sterilised by shaking for 1 min in a solution of 10 % v/v Domestos bleach (Lever Fabergé Ltd., Kingston-upon-Thames, UK; www.domestos.co.uk), rinsed and soaked in purified water for 1 h. Five seeds were incubated at 30°C on filter paper moistened with purified water in Petri dishes. Seeds were inspected frequently to replenish water and any germinating seeds immediately transferred to compost. Plants were grown in pots using an equal mixture by volume of John Innes No. 3 compost (William Sinclair Horticulture Ltd., Lincoln, UK; www.william-sinclair.co.uk) and Levington M3 compost [The Scotts Company (UK) Ltd., Ipswich, UK] under glasshouse and controlled environment room conditions described in Section 3.7. Water was applied on an *ad lib* basis (at about 3 d intervals) as and when the compost showed signs of being dry. Nutrients were replenished occasionally through irrigation using Poly-Feed fertilizer (18:18:18+2) [Haifa Chemicals (Ltd.), Haifa Bay, Israel] at a normal feed of 1.0 g l^{-1} when plants showed some signs of nutrient stress. Perpetuation of clonal stock plant material was maintained by clonally propagated plants (rooted stem cuttings and *in vitro* generated plants).

Occasional examination for insects/pests was carried out and several insect pests were observed and controlled through biological means and in some cases by use of chemicals. Red spider mites (*Tetranychus urticae)* and thrips (*Frankliniella sp.)*

were effectively controlled by application of predators (*Phytoseiulus persimilis*) and (*Amblyseius cucumeris*) (Syngenta Bioline, Little Clacton, UK) respectively, every 14 d until the infestation was under control. Aphids were controlled by the use of the systemic chemical Imdacloprid (Intercept; Monro South, Wisbech, UK; www.monrosouth.co.uk) applied to soil/growth medium and taken up by roots. Scale insects were also observed, but no control measure was applied as infested plants were destroyed.

3.4 *In vitro* propagation experiments

3.4.1 Preparation of stock solutions of plant growth regulators (PGRs)

PGR stock solutions for *in vitro* trials were made mostly at a concentration of 0.5 mg ml^{-1} for the auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA), cytokinins 6-benzylaminopurine (BAP), 6-(γ,γ-dimethylallylamino)purine (2iP), thidiazuron or N-phenyl-N′-1,2,3-thidiazol-5-ylurea (TDZ), anti-auxin/auxin polar transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) and the gibberellin GA_3. Anti-gibberellins/gibberellin biosynthesis inhibitor paclobutrazol [N-dimethylaminosuccinamic acid (PP333)] was made at 0.02 mg ml^{-1} because of its low solubility in water. To prepare 100 ml of PGR stock solution of 0.5 mg ml^{-1} concentration, 0.05 g of the pure PGR was placed in a small beaker with a magnetic stirrer and 1-3 ml of 1 M KOH added to dissolve the compound. While continuously stirring, water was added very slowly with the aid of a burette and volume finally made up to 100 ml with a volumetric flask. To prepare a stock solution of PP333, 10 mg was placed in a beaker and dissolved, as for the other PGRs, and volume made up to 500ml with purified water. All stock solutions were kept in the refrigerator at 4°C.

3.4.2 Preparation of culture media

Most growth media used in this study were made from MS medium (Murashige and Skoog, 1962) powdered salts (DUCHEFA, Haarlem, The Netherlands) at a concentration of 4.41 g l^{-1}, unless otherwise stated. In the preparation of media, the following were added into half volume of purified water, namely sucrose (Fisher Scientific UK Ltd., Loughborough, UK; www.fisher.co.uk) at 30 g l^{-1}, MS salts and PGRs. The volume of the solution was made up with purified water and the pH

adjusted to 5.6-5.8 by adding 0.1M or 1M HCL or KOH, before adding the gelling agent, agar (Sigma-Aldrich Co, St. Louis, USA) at 7 g l^{-1}. The medium was autoclaved for 10 min at 106°C to melt the agar and thereafter dispensed while hot into screw top glass vessels using an Accuramatic - MK5 dispenser (Accuramatic, Wellington, UK). The medium in the culture jars was sterilised by autoclaving at 120°C (15 psi) for 20 min.

Heat labile media components such as TIBA and PP333 were filter sterilised using a Sartorius Minisart NML single use syringe filter unit with pore size of 0.20 μm (Sartorius AG 37070, Goettingen, Germany). The required volume of sterilised heat labile components was then added to sterilised medium after it was allowed to cool to 40°C and the required volume of medium dispensed aseptically into sterile culture jars.

3.4.3 Aseptic technique

All aseptic procedures were carried out in a laminar flow cabinet that had been thoroughly sterilised with 70 % (v/v) Industrial Methylated Spirit (IMS) to minimise contamination. Metallic instruments, such as scalpel blades and forceps were sterilised by heating in a hot glass bead sterilizer (Model Steri-350, Steripot; Simon Keller AG, Burgdorf, Switzerland; www.simonkeller.ch) at 250°C for ≥1 min. Hands, surfaces of culture vessels and the laminar flow cabinet were regularly disinfected by spraying with 70% IMS. Only sterilized material was placed inside the lamina flow cabinet.

3.4.4 Preparation, inoculation and incubation of cultures

Explants used for establishment of axenic cultures were nodal segments obtained from stock plants growing in glasshouses or controlled environment rooms. The nodal segments were each cut into length of about 2.5 mm (Plate 3.2), surfaced sterilised in 10 % (v/v) Domestos bleach for 20 min and then rinsed 3 times with sterile purified water. Each explant was trimmed to 10-15 mm by removing sterilant-damaged ends before inoculation/insertion onto culture medium in an incubation vessel. Cultures were incubated in glass vessels (60 and 100ml capacity) with metal screw tops. Sterilization and inoculation of explants were conducted in the laminar

flow bench under aseptic conditions and cultures incubated in a soil-free controlled environment room as described in Section 3.7.1. Cultures were frequently inspected to detect and dispose off any contaminated material.

Plate 3.2 Stem nodal segment with axillary bud (micropropagule) used as explant for *in vitro* propagation (micropropagation). Bar = 15 mm.

3.5 Propagation by stem cuttings

3.5.1 Preparation of auxin solution

IBA potassium salt was used as auxin treatment to stimulate adventitious root formation. The stock solution was prepared in the highest concentration required for the experiment and then weaker solution made by dilution of the stock solution. Since less quantities of solution were usually required, the greatest volume that could be prepared was 50 ml. To make a solution of 10 % auxin concentration, 5 g of potassium salt was placed in a beaker with a magnetic stirrer and dissolved slowly by adding purified water. The solution was stirred continuously and the volume finally made up to 50 ml using a volumetric flask. To prepare 50 ml of 5% auxin concentration, 25 ml of the stock solution (10% auxin) was measured and the volume made up to 50 ml with purified water and the same dilution procedure followed for other concentrations. Solutions were stored in refrigerator set at 4°C.

3.5.2 Preparation and incubation of propagules

Stem segments with one fully developed healthy trifoliate leaf (Plate 3.3) were used as propagules. Stem segments were cut into sections of about 10 cm in length and auxin applied by dipping about 2 cm of stem basal end in the auxin solution for about 60 s. About 2.5 cm stem of auxin treated cutting was inserted into the rooting

compost made up of equal parts by volume of Perlite (Silvaperl Graded Horticulture Perlite; William Sinclair Horticulture Ltd.) and peat (Scott International B.V., Geldermalsen, The Netherlands) placed in Plantpak plastic seedlings trays with 50 cells each (Desch Plantpak Ltd., Maldon, UK; www.desch-plantpak.co.uk). Each cell in the tray had a capacity of 35 cm^3. Trays holding cuttings were placed in unheated polyvinyl propagators with transparent lids (Base, 41×51×8 cm; lid, 15 cm height) (Richard Sankey, Nottingham, UK; www.rsankey.co.uk) to maintain humidity and incubated in controlled environment growth rooms as described in Section 3.7.2. Cuttings were sprayed every 3 d with a mist of water to maintain humidity around the cuttings.

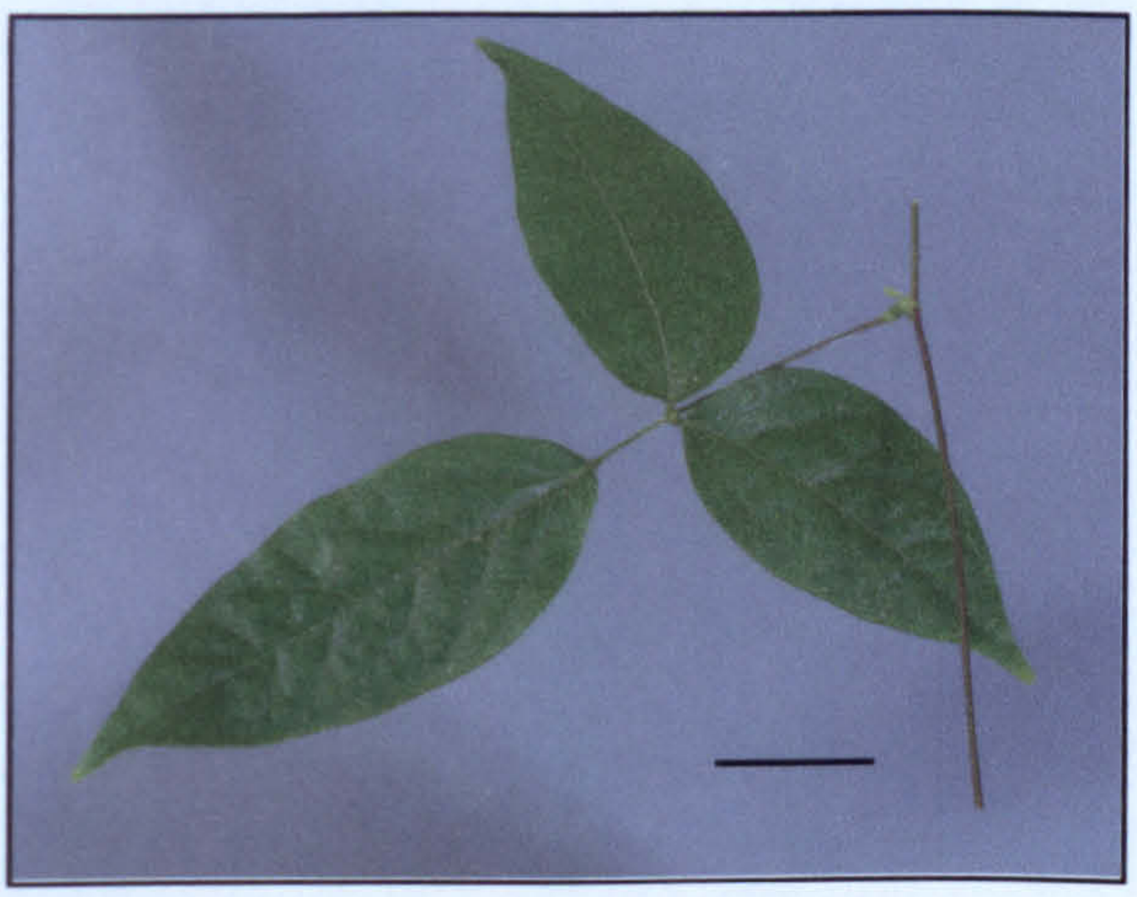

Figure 3.3 Stem nodal cutting with an axillary bud used as propagule for rooting (macropropagation) (Bar = 20 mm).

3.6 Nodulation and nitrogen fixation

3.6.1 Rhizobial inocula

Inocula used were prepared from strains obtained from various sources as listed in Table 3.2.

Table 3.2 Rhizobial strains used in experiments

Strain Host range	Host plant	Source
Bradyrhizobium sp. AUEB20	*Erythrina brucei*	Prof. D Kleiner, Microbiologie der Universitat, Bayreuth, Germany
Bradyrhizobium sp. CP279 (broad host range)	*Parasponia andersonii*	Prof. BG Rolfe, The Australian National University, Canberra, Australian.
Rhizobium sp. NGR234 (broad host range)	*Lablab purpureus*	Prof. BG Rolfe, as above
Rhizobium sp. ORS302 (*Aeschynomene* spp.)	*Aeschynomene pfundii*	Dr. A Alazard, ORMSTON, Dakar, Senegal.
Rhizobium sp. ANU240 (broad host range)	*Lablab purpureus*	Prof. BG Rolfe, The Australian National University, Canberra, Australian.

3.6.2 Cultivation of rhizobia

Bradyrhizobium AUEB20 strain was cultivated on yeast extract mannitol medium [YEM; mannitol (10 g), yeast extract (0.5 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), NaCl (0.2 g) and purified water 1 l] for slow growing bacteria, according to Assefa and Kleiner (1997), while the other rhizobial strains were cultivated on YEM without KH_2PO_4 but with yeast extract and NaCl reduced to 0.4 and 0.1 g, respectively (Dalton, 1980). For the preparation of semi-solid medium, agar was added at 20 g for *Bradyrhizobium* AUEB20 strain and 15 g for other rhizobial strains. The medium was sterilised in the autoclave at 120°C (15 psi) for 20 min and dispensed under aseptic environment into sterile Petri dishes at a volume of 30 ml each. Cultures were grown first in semi-solid medium in the dark at 30°C ±1 for multiplication. Stock cultures were kept in the refrigerator at 4°C and subcultured every 28 d for maintenance.

3.6.3 Preparation of inocula, inoculation and cultural care of plants

For preparation of inoculum, a Petri dish of semi-solid culture was divided equally into 2 and each half transferred to 150 ml liquid medium (broth cultures) and incubated in the dark on a shaker for 24 h. The broth culture was diluted to 50% of its original strength with liquid medium and number of bacteria (Inoculum strength) in the inoculum was estimated by the dilution plate count method (Alexender, 1999). AYB1 and AYB2 plants raised through stem cuttings were inoculated as soon as they

formed roots by immersing the roots in the inoculum and then potting in low nutrient compost made of equal parts by volume of Perlite and Levington F2s compost [The Scotts Company (UK) Ltd.] for all the experiments. Thereafter, the remaining inoculum was evenly distributed among the plants. Pots were placed in Stewart plastic trays (100×40×5 cm) to trap inoculum-contaminated water. Irrigation was with tap water on an *ad lib* basis from below by capillary to avoid leaching of the inoculum. Pest management was practised according to the procedure described in Section 3.3.

3.6.4 Detection of nitrogen fixation by the Acetylene Reduction Assay (ARA) for nitrogenase activity

The ability of nitrogenase, the enzyme responsible for reduction of nitrogen gas to ammonium (NH_4), to act on other substrates with a triple bond makes it possible to be assayed by gas chromatography. The ARA utilises industrial acetylene gas (C_2H_2), which is reduced by nitrogenase acting on the triple bond to give ethylene (C_2H_4). The ethylene produced is assayed by gas chromatography giving an indication of the nitrogenase activity in the nodules hence the fixation capacity/efficiency.

Nodules were detached from newly harvested intact plants and assessed for nitrogenase activity by using the ARA procedure described by (Hardy *et al.*, 1968) and (Turner and Gibson, 1980). Nodules were transferred into 70 ml tubes and sealed with gas tight rubber closures (Subaseals^{TM}; Scientific Laboratories Supplies, Nottingham, UK); 10% (7 ml) of air was removed with a hypodermic needle and replaced with the same volume of industrial acetylene gas. Another tube was filled with fresh roots from freshly harvested intact plants of the control treatment (i.e. plants that did not receive any rhizobia and thus did not nodulate) and given acetylene as above to serve as control. Tubes were incubated at 25°C for 1 h and, thereafter, 0.5 ml samples of gas from each tube was removed with a hypodermic needle and analysed for ethylene production with a Pye Unicam PU4500 gas chromatography (GC) (Pye Unicam Ltd. Cambridge UK) with 183 cm (2.0 mm i.d.) glass column containing "Porapak N" with a mesh size of 80-100 (Phase Separations Ltd., Watford, UK). The carrier gas was nitrogen:hydrogen:air (15:17:300 by volume) at flow rate of 27 ml min^{-1}. The oven containing the column was set at 60°C

while flame ionisation detection was set at 121°C. The flame ionisation detection range (sensitivity) was set at 256.

Total ethylene generated was calculated using conversion factors (Table 3.3) from the calibration curve for the Pye Unicam PU4500 GC (Webster, 1995; Stone, 2000). The peak height from the chart was determined using a ruler and C_2H_4 produced calculated using values given in Table 3.3. The number of nmoles present in a sample was calculated as (e.g. at a sensitivity detection range set to 256):

A 1 mm peak height = (2.28/100) × 2 × head space volume.

Where head space volume refers to the volume of the tube holding the sample (70 ml in the case of this study).

Table 3.3 Conversion factors for calculating ethylene concentration (Adapted from; Stone, 2000)

Detector Range	nmoles of ethylene
64	0.57
128	1.14
256	2.28
512	4.56

3.6.5 Quantification of amount of N_2 fixed (tissue nitrogen concentration analysis)

Whole above plant samples were dried at 80°C for 48 h and ground to a fine powder using CYCLOTEC 1093 Sample mill (FOSS TECATOR, Höganäs, Sweden). Determination of total tissue nitrogen was carried out on 55-60 mg of finely ground sample using a NA 200 Nitrogen Analyser (Fisons Instruments, Okehampton, UK). The instrument was designed for the macro determination of total nitrogen present in a wide range of organic and inorganic samples. The analytical method is based on the complete and instantaneous oxidation of the sample by "flash combustion", which converts all organic and inorganic substances into combustion products. The resulting combustion gases pass through a reduction furnace and are swept into the GC column by carrier gas (helium). The gases are separated in the column and detected by the thermal conductivity detector (TCD) that gives an output signal proportional to concentration of the nitrogen of the mixture.

Nitrogen was obtained by comparing the values obtained from the analysis of the sample with the analysis of a suitable standard. The following equation was used in calculation of nitrogen percentage:

N% = K × (I – b)/WS, where K = Average factor, I = Sample integral, W = Weight of sample in mg, B = blank

K factor calculation: K = Th% × WS/I – b, where Th% = Theoretical percentage of standard, WS = Weight of standard in mg, I = Standard integral (peak area of standard), B = Blank (important in trace analysis)

3.6.6 Chlorophyll estimation

Chlorophyll in the leaves was estimated following the method established by (Inskeep and Bloom, 1985) which employs *N,N*-dimethylformamide (DMF) as solvent for the extraction of chlorophyll. Fully developed leaves were selected randomly from vines at the top of the plant and cut into small discs using a cork borer. The leaf discs were weighed (aiming for 0.1-0.2 g sample), diameter measured and leaf area calculated before immersing them in 10 ml DMF in 125 ml conical flasks. The flasks were sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan), covered with aluminium foil to exclude light and shaken for 24 h at 20°C. Due to eroding effect of DMF on plastic compounds, instead of using automatic sampler in the spectrophotometer, precision optical glass cuvettes of 3.5 ml volume (Hellma CXA 145.040C, Fisher Scientific UK, Loughborough, UK. www.fisher.co.uk) were used to hold chlorophyll samples. A cell attached to automatic sampler was gently lifted out of the cell holder of the spectrophotometer (Pye Unicam SP6-500 UV; Pye Unicam Ltd., Cambridge UK) and glass cuvettes holding 3 ml of chlorophyll extracts inserted into the cell holder. The compartment lid was closed and the optical density (OD) read at 647 and 664.5 nm. The chlorophyll concentration of the sample leaves (mg l^{-1}) was estimated using the following equations:

Total chlorophyll $= 17.9*A_{647nm} + 8.808*A_{664.5nm}$

Chlorophyll a $= 12.7*A_{664.5nm} - 2.79*A_{647nm}$

Chlorophyll b $= 20.7*A_{647nm} - 462*A_{664.5nm}$

(A=absorbence)

The resulting value was divided by 100 or 1000 depending on the volume of the solvent used to obtain the total quantity of chlorophyll extracted from the leaf sample (mg). Total chlorophyll was then divided by fresh weight of sample disc to give chlorophyll content in mg g^{-1}.

3.7 Controlled environment facilities

3.7.1 Tissue culture growth rooms

Cultures were incubated in a controlled environment room set at a constant temperature of 26°C ±1 with a 12 h photoperiod and light intensity of 75-80 µmol m^{-2} s^{-1} provided by warm white fluorescent tubes (MCFE 65-80W/29-30, Philips, Eindhoven, The Netherlands).

3.7.2 *In vivo* plant control environment growth rooms

Conditions in the growth rooms closely mimicked those in the tropics where the species grows.

Nodulation Experiment 2 and stock plants	-	25°C night and 30°C day
Rooting of stem cuttings and stock plants	-	25°C night and day

The photoperiod was set for 12 h and provided by 400 Watts high pressure mercury vapour lamps (HLRG; Philips, Eindhoven, The Netherlands) giving a light intensity and irradiance of 450±25 µmol m^{-2} s^{-1}.

3.7.3 Glasshouse

Temperature in the glasshouse was controlled by motorised valve regulated by a thermostat. The temperature was maintained within 27°C ±3, above which the top ventilators opened to provide cooling and below which the heating system would be triggered to provide heat supplied by low pressure hot water circulated in pipes around the glasshouse perimeter. At the onset of shorter days (from October to April), supplementary lighting was provided by 400 Watts high pressure sodium lamps providing a total irradiance of 40 Wm^{-2} to maintain a photoperiod of 12 h.

3.8 Plant growth measurements

3.8.1 *In vitro* growth measurements

Measurements taken varied with experiments but generally comprised of shoot height, shoot fresh weight, number of nodes, root number, length of the longest root, root emergence rate, rooting percentage and callus size (weight and diameter). Callus diameter was estimated by taking an average of the longest and shortest spread of the callus material on substrate and weight determined from fresh friable/soft callus material thus excluding the corky tissue formed by the original explants. A bud was scored as a shoot if it measured 10 mm or longer. Shoot height was measured of the longest shoot by taking the distance between the point of shoot attachment on the original explant and the apex. Height measurement was taken while a shoot was still in the culture vessel to avoid contamination.

3.8.2 Growth measurements of *in vivo* grown plants

Growth parameters measured were leaf number, leaf area, stem area and dry weights of stems and leaves. Plant material was dried at 80°C for 48 h to determine absolute growth, while leaf and stem areas were measured with a leaf area meter (Model LI-3100 Area Meter; LI-COR. Inc., Lincoln, USA).

3.9 Experimental design and data analysis

Most experiments were conducted as a completely randomised design (CRD). Data was subjected to analysis of variance (ANOVA) using Genstat Release 6 and 7 (Lawes Agricultural Trust, Rothamsted, UK) that provided means, standard errors of differences between means (*s.e.d.*) for all variables. Comparisons were performed at 5% level of significance.

CHAPTER 4

MORPHOLOGY AND GROWTH

4.1 Introduction

AYB is an important crop in most parts of West and Central Africa relished for its protein-rich small tuberous roots and good yields of edible seed (National Academy of Sciences, 1979). Its leaves are utilized as a spinach/cooked vegetable (Tindall, 1983). It is one of three taxa used by humans from the large genus, *Sphenostylis* E. Meyer (Leguminosae: Papilionoideae: Phaseoleae) and the most widely distributed and morphologically variable species in the genus although cladistic analysis of morphological and chloroplast DNA (cpDNA) support inclusion of these elements in a single species (Potter, 1992).

Various disciplines aimed at increasing knowledge of plants are either based on plant morphology or are closely related to it (Claben-Bockhoff, 2001). Thus, the practical relevance of plant morphology, including morphogenesis, can be seen in all disciplines of plant biology (Sattler and Rutishauser, 1997). Although not detailed, observations made on stock plants kept in different conditions during this study have also confirmed variability between the landraces regarding their morphology as well as response to the environment. However, detailed research on the species growth and development has not been undertaken and thus very little documented information is available.

Initially, plants were grown to try to produce seeds and kept in controlled environment rooms at 25/30°C (night/day) and a 12 h photoperiod under the tropical climatic conditions from which the landraces originate. Later, when these plants failed to produce flowers and propagation by cuttings was adopted, day temperature was adjusted from 30°C to 25°C to be conducive for the rooting of cuttings. Some stock plants (AYB1, AYB2, AYB3 and AYB5) raised from seeds were also kept under these same conditions to utilise the space that was available. Observations on the morphology and growth of these plants were made and also on plants grown in the glasshouse (both in soil and pots) during the summer periods.

4.1.1 Seed germination and emergence

AYB landrace seeds vary in size and colour of seed coat (Plate 4.1). Due to the inadequate supply and poor quality (low viability) of seeds that were available, it was not possible to make detailed observations on germination. However, seed germination was found to be hypogeal with seedling emergence taking >7 d.

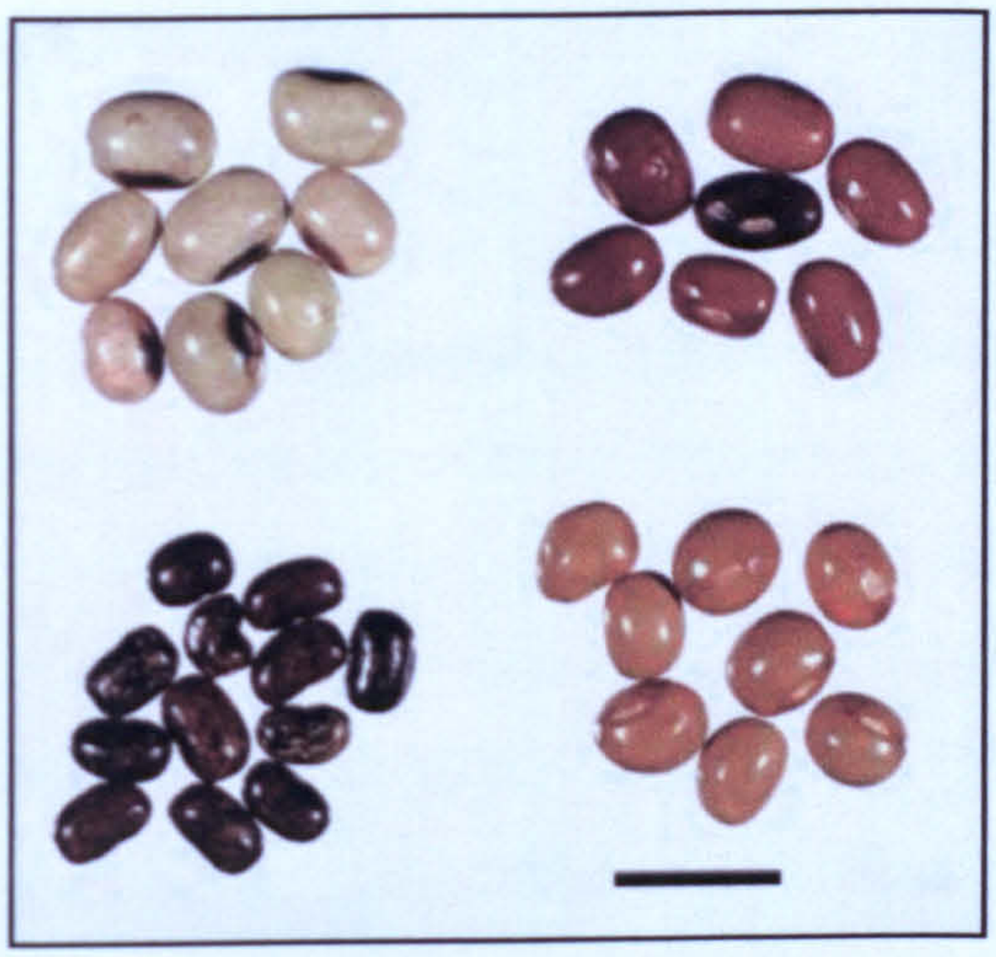

Plate 4.1 Variability in seeds of AYB landraces. Bar = 10 mm.

4.1.2 Root growth

The plants grown in pots and directly in open beds possessed roots with few or no root hairs, but directly in open beds, they were highly branched and grew to considerable depth (Plate 4.2 A). There was no distinct tap root because the plants used were raised from cuttings that only possessed adventitious roots. It was also found that AYB formed adventitious roots easily when trailing stems contacted the soil (Plate 4.2 B). While other landraces did not develop tubers, AYB5 plants grown at 25°C and 12 h photoperiod produced sweet potato or yam-like tubers (Plate 4.2 C & D). Nodules were not formed on the roots of all the landraces grown without bacterial inoculation under any of the conditions described, including those grown in the soil in the glasshouse.

The extensive fibrous root system observed on plants grown in the soil revealed AYB's potential to withstand drought by exploring water in deeper soil layers. Although plants in both experiments were watered on an *ad lib* basis, plants grown in pots experienced relatively more leaf drop than those grown in the soil, possibly due

to water stress as a consequence of restricted root growth. Although not known to be grown for tubers, plants of AYB5 formed tubers, whereas tuberisation was not observed in other landraces, including AYB3, which was collected from Congo/Zaire where it is known to be cultivated for its tuberous roots. This observation could be attributed to various factors such as genotype, environment or their interaction. Although mostly grown for seeds in West Africa, AYB is also known to form tubers which serve as organs of perennation (Potter, 1992). Information has not been found on the effect of environment on tuberisation of AYB. In major areas of production in Zaire, tubers are usually harvested 7 to 8 months after plants are propagated by seed (Potter, 1992). Studies conducted under tropical conditions with yam bean (*Pachyrhizus* spp.), a tuberous legume from South America, indicated tuberisation to be photothermoperiod insensitive, starting 4-6 weeks after germination although development exclusively under short days adversely affected tuber yield due to restricted shoot (photosynthetic apparatus) development required for root enlargement later (Sorensen *et al.*, 1993). Sweet potato (*Ipomoea batatas*), although not a legume, is one of the tropical tuber crops known to be sensitive to short days and low temperature (Onwueme, 1978).

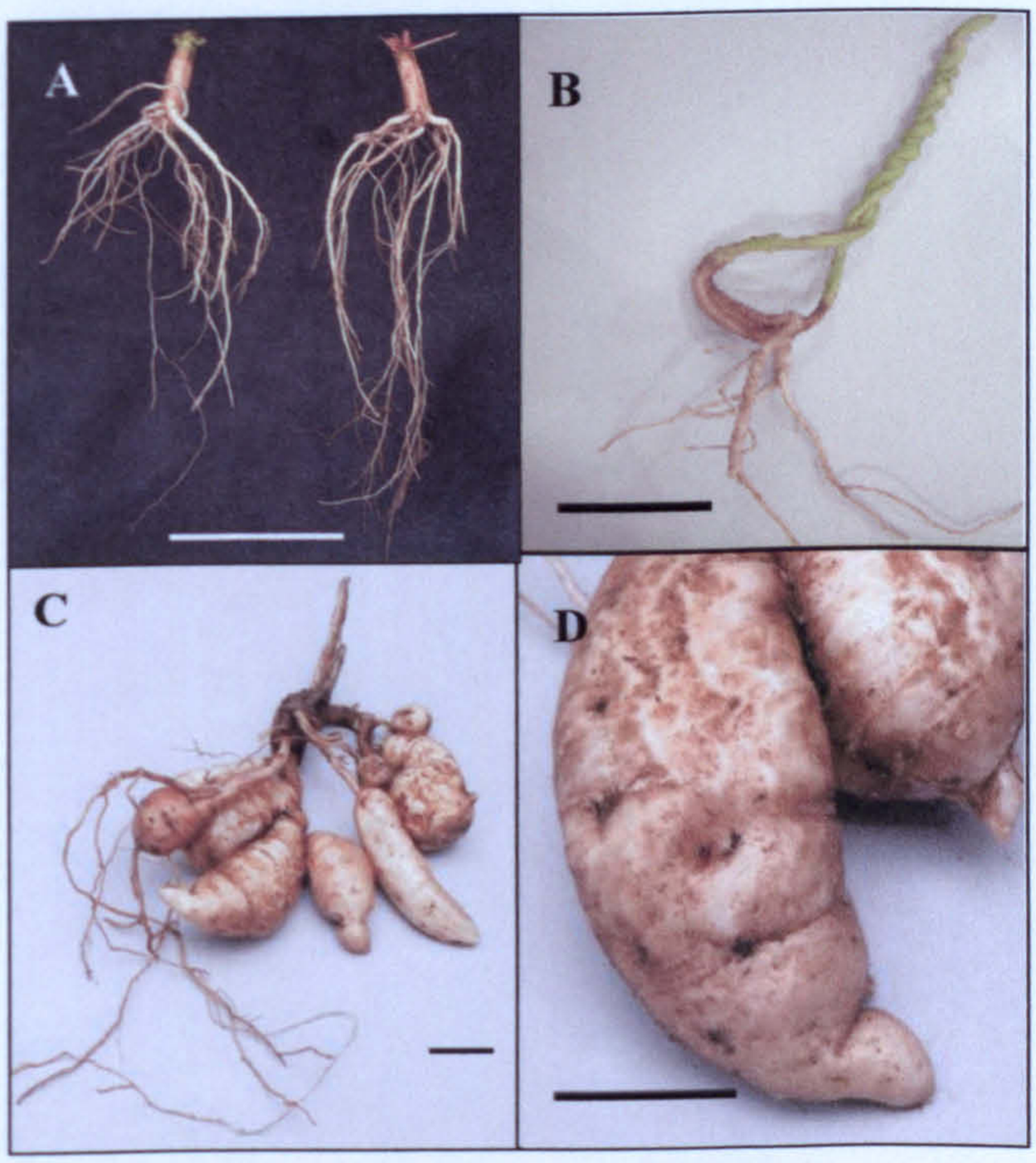

Plate 4.2 (A) Extensive roots of AYB landraces (left, AYB1 and right, AYB2) Bar = 150 mm. (B) Adventitious roots growing on AYB1 stem trailing on the ground (layered stem). Bar = 40 mm. (C & D) Sweet potato like tubers formed on AYB5 plant. Bars = 20 mm.

4.1.3 Vegetative structures

Plants from both AYB1 and AYB2 landraces produced abundance of twining vines that grew through the vents in the roof of the glasshouse where they were estimated to exceed 4 m height (Plate 4.3). The main stem feature differentiating AYB landraces was the stem colouration with AYB1 being light green and AYB2 being red in colour (Plate 4.4). The landraces also differed in internode length; AYB2 had longer internodes than AYB1 (Plate.4.4). Consequently, AYB1 usually had more leaves per vine than AYB2 and, hence, more leaves per plant. The leaves were trifoliate and varied in size but, when fully grown, leaflets exceeded 8 cm in length and 4 cm in width (Plate 4.4). AYB2 leaves were slightly larger than those of AYB1.

Plate 4.3 Vigorous growth habit of AYB plants 112 days after transplanting. Bar = 1 m.

Plate 4.4 Variation in stem colour and internode length of AYB landraces (left: AYB1 and right: AYB2). Bar = 40 mm.

4.1.4 Reproductive structures

Flowering occurred in AYB1, AYB2 and AYB5 growing at 25°C (night and day) 7 months after sowing, whereas there was no reproductive development after more than 12 months in all of the landraces grown at a higher day temperature (30°C). The racemose inflorescences arose from the reproductive buds found in the axils of leaves alongside vegetative buds and were comprised of 2 or more pink to magenta coloured bisexual flowers arranged monopodially such that the pods developed and matured at different times (Plate 4.5). Reproductive buds were small with numerous oval structures that were possible flower buds, as opposed to the pointed apex of vegetative buds with structures that were possible leaflets of a trifoliate leaf (Plate 4.5A). Fully developed pods measured >20 cm in length and matured into non-shattering woody pods that contained up to 20 seeds (Plate 4.6). This reproductive growth was observed to be continuous with some signs of disruption in vegetative growth as the heavily bearing plant displayed a less prolific vegetative growth possibly due to competition for assimilates (Plate 4.7). Amongst the pod bearing landraces, AYB5 was the most prolific, bearing flowers and pods profusely.

Flowering of AYB landraces was not realised for most of the study period when plants were grown under conditions that were thought to be similar to the species natural habitat of the tropics (30°C and 12 h photoperiod). However, some of the plants flowered 7 months after planting when grown at 25°C with a 12 h photoperiod, and also continued to grow vegetatively. Nigerian genotypes, though

slow to set seeds, are reported to flower and form pods continually all year-round (National Academy of Sciences, 1979). There is little information available on the control of flowering in AYB, but there has been a suggestion that AYB might be photoperiod sensitive. Tindall (1983) indicated that AYB might be photoperiod sensitive, while Okpara and Omaliko (1997), suggested a photoperiodic response to short days. The time of planting affected onset of flowering with early sowing dates (mid-May to mid-June) causing a delay of up to 2 weeks, while plants sown late (mid-July to late-July) flowered within 13 weeks (Okpara and Omaliko, 1997). The development pattern of yam bean (*Pachyrhizus* spp.) under tropical conditions has shown the species to be short-day sensitive where development under long-days is characterised by shoot growth with formation of many vine-like shoots and flower initiation occurring only when the day-length decreases and approaches 12.5 h (Sorensen *et al.*, 1993). Sweet potato is one of the tropical root tuber crops sensitive to short days which flowers frequently when the photoperiod is <11 h and fails to flower >13.5 h (Onwueme, 1978). As a tropical root tuber and seed producing plant, AYB may follow a developmental pattern similar to those displayed by the tropical root tuber crops mentioned above.

Although these reports do not refer to temperature effects, it is possible that temperature or the interaction of temperature and photoperiod may also play a role. A reduction in natural photoperiod is usually concomitant with a reduction in temperature such that flowering is enhanced under short day/lower temperature conditions. This could explain why the AYB landraces used in this study did not flower when they were grown at 30°C, but flowered when grown at a day temperature of 25°C. While flowering in bambara groundnut (*Vigna subterranea*) can be day neutral or show some short day response, some genotypes have been reported to flower only at 20 and 25°C, but not at 30°C (Linnemann and Azam-Ali, 1993). Therefore, AYB response might be similar to that of the bambara groundnut genotypes mentioned here.

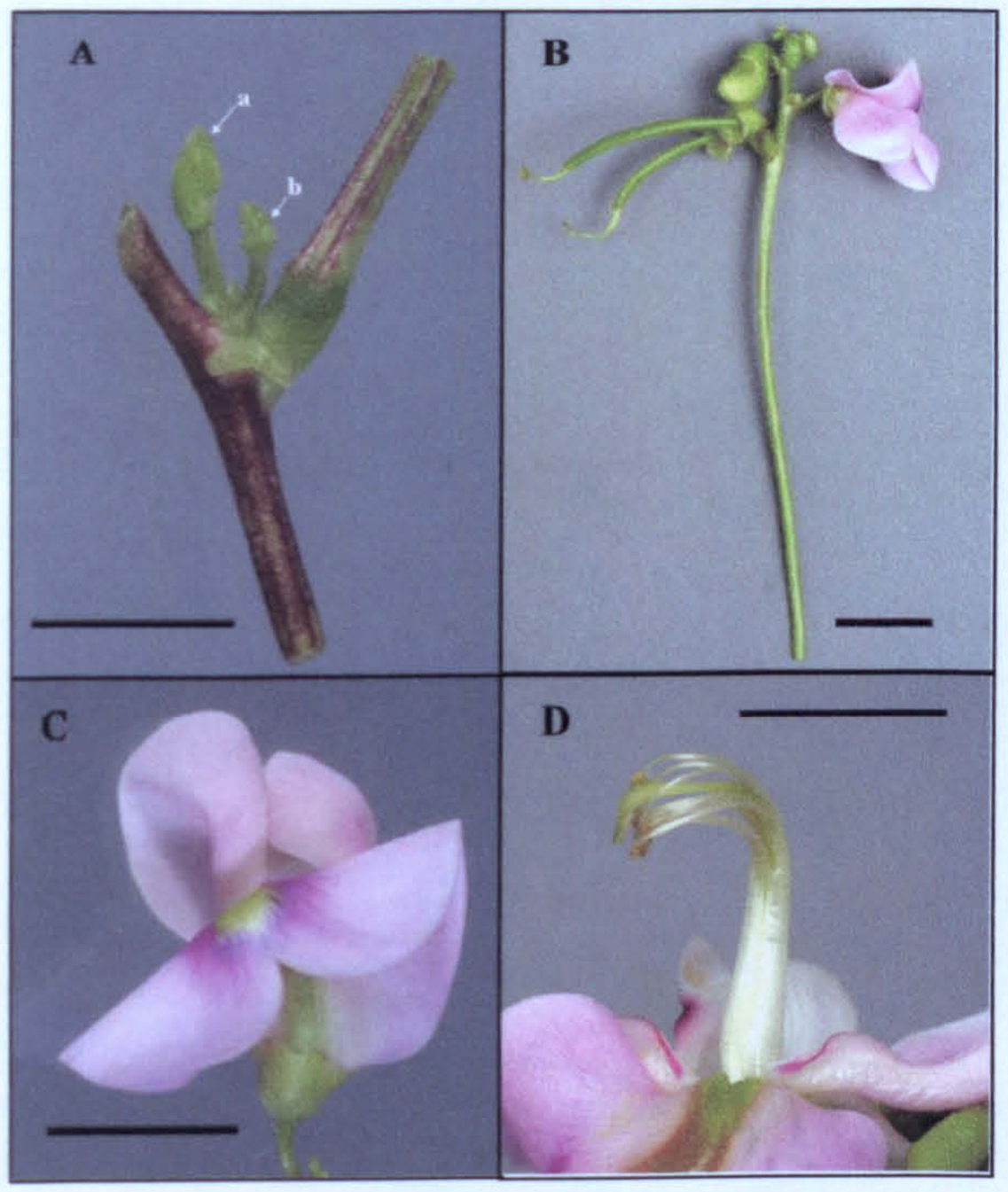

Plate 4.5 Stem nodal segment (A) bearing a vegetative bud (a; long and pointed) and a reproductive bud (b; short and round); gives rise to racemose inflorescence (B) bearing young developing pods, brightly coloured bisexual flower (C, D). Bars = 10 mm (A), 20 mm (B, C, D) of AYB5 landrace.

Plate 4.6 Pods produced by AYB plants (A) immature and (B) mature. Bar = 20 mm.

Plate 4.7 AYB5 plant producing pods profusely as well as growing vegetatively. Bar = 30 cm.

4.2 Growth of AYB landraces in pots and in the soil

The paucity of information on the morphological development of AYB makes efforts aimed at broadening scientific knowledge very difficult. This study here focused on AYB's external form and structure, to establish changes with time and to characterise variability between landraces. The aim of the study was to test the hypotheses that AYB landraces will grow at different rates and reach maturity (flowering and seed production) at different times; and that landraces differ in yield (dry matter and seed). The objective was to identify morphological characters of AYB that delimit the AYB landraces with reference to plant growth. The study was conducted in two separate trials using two landraces (AYB1 and AYB2) with plants raised through rooted stem cuttings, growing plants in pots filled with a 50:50 by volume mixture of

John Innes No. 3 compost and Levington M3 compost and growing plants directly in open beds.

Two glasshouse experiments were carried out to characterise the growth of AYB landraces. In Experiment 1, plants were grown in pots from July to October 2003 while in Experiment 2 they were in the ground from April to August 2004 (Plate 4.8). In both experiments, plants of AYB1 and AYB2 raised from cuttings were used. Curves best fitting each data set were superimposed according to Clewer and Scarisbrick (2001) to characterise the growth model followed in each experiment.

4.2.1 Materials and methods

Experiment 1: Pot experiment

Plants were grown in pots in a glasshouse under conditions of natural light supplemented with 400 Watts high pressure sodium lamps (Chapter 3, Section 3.7.3 to maintain a minimum of 12 h photoperiod as the days shortened. Rooted cuttings were kept for 4 weeks under nursery conditions and transplanted into 19 cm Plantpak plastic pots after acclimation to start the trial. Midway into the experimental period (at 6 weeks), the plants were repotted into 25 cm Plantpak plastic pots. A 50:50 by volume mixture of John Innes No. 3 compost and Levington M3 compost was used as growing medium throughout the trial. The experiment was set up as a complete randomised design with 3 replications (3 plants) and 5 sampling stages. Five destructive harvests of the shoots (above ground parts) were taken every 21 d from the date of transplanting and growth parameters (leaf number, leaf area, average leaf size, leaf dry weight, stem area, stem dry weight, total green (shoot) area and total shoot dry weight) measured according to the procedures described in Chapter 3, Section 3.8.2. Relative growth was computed according to the procedure for growth analysis described by Hunt (1982).

Experiment 2: Soil experiment

Plants were grown in the glasshouse soil with a basal fertilizer of nitrogen, potassium [NH_4NO_3 (34.5% N; ICI Chemicals & Polymers Ltd., Billingham, UK)], [KNO_3 (13% N and 46% K_2O; Haifa Chemicals, Haifa Bay, Israel)] and phosphorus [J. Arthur Bower's Superphosphate (7.4% P_2O_5); William Sinclair Horticulture Ltd., Lincoln, UK)] incorporated at 45 kg ha^{-1}. Rooted cuttings were kept for 4 weeks

under nursery conditions (low light and cool temperature conditions) before transplanting at a spacing of 80 × 80 cm. The experiment was set up as a randomised block design with 5 blocks and 5 plants per landrace in each block. Destructive harvests of 5 plants per landrace were made with the first harvest 28 d after transplanting (DAT) and subsequent harvests at 21 d intervals. At each harvest, 1 plant was selected at random from each of the 5 blocks and the parameters listed in Experiment 1 measured according to the procedures described in Chapter 3, Section 3.8.2. Extensive growth of AYB plants (Plate 4.9) at the time of the fifth harvest made it difficult to separate the plants and hence this final harvest was impractical.

Plate 4.8 Experimental layouts for Experiment 1 (A) and Experiment 2 (B). Bars = 40 cm (A), 80 cm (B)

Plate 4.9 Extensive entangled stem growth of AYB landraces at 112 d from transplanting. Bar = 30 cm.

4.2.2 Results

As flowering was not observed in any of the two landraces during these growth trials, data for reproductive growth parameters is not available. Only data on vegetative parameters is presented. Growth parameters in the pot experiment showed characteristic power growth model, while an exponential growth was displayed in the soil experiment with growth going through an initial slow phase and then a rapid phase characterised by a sudden peak. The experiments were terminated before reproductive development and senescence.

4.2.2.1 Vegetative growth

4.2.2.1.1 Leaf growth

In Experiment 1, there were significant differences in the number of leaves between the two landraces at 42 DAT ($F_{(1,4)}$ = 8.43, p<0.05) and 84 DAT ($F_{(1,4)}$ = 20.78, p<0.01), with AYB1 possessing more leaves than AYB2 (Figure 4.1). Although differences were not significant at other harvests, AYB1 still had relatively more leaves than AYB2. In Experiment 2, although the differences were not significant at all growth stages, AYB1 appeared to have more leaves than AYB2 at 28 and 49 DAT, while AYB2 had more than AYB1 at 70 and 91 DAT (Figure 4.2).

In Experiment 1, the trend for leaf area was the same as that for leaf number with AYB1 having relatively larger leaf area than AYB2 at all stages, but with a highly significant ($F_{(1.4)}$ = 61.48, p <0.001) difference at 84 DAT (Figure 4.3). In contrast, in Experiment 2, AYB2 appeared to be more vigorous than AYB1 although the difference was not significant at any stage (Figure 4.4). AYB2 had relatively larger leaves than AYB1 in both experiments, but the difference was only significant at 42 DAT ($F_{(1.4)}$ = 51.58, p<0.01) and 105 DAT ($F_{(1.4)}$ = 10.81, p<0.05) in Experiment 1, while in Experiment 2 it was significant ($F_{(1.4)}$ = 11.16, p<0.05) at 28 DAT (Figures 4.5 and 4.6). Although there was a change in average leaf size in Experiment 2 from 10 cm^2 to >30 cm^2, this did not exceed 40 cm^2 recorded at the beginning of Experiment 1. The average leaf size for both landraces stabilized at <40 cm^2 in both experiments. Leaf drop occurred more in Experiment 1 than in Experiment 2 for both landraces, possibly due to the restriction of root growth imposed by the pots.

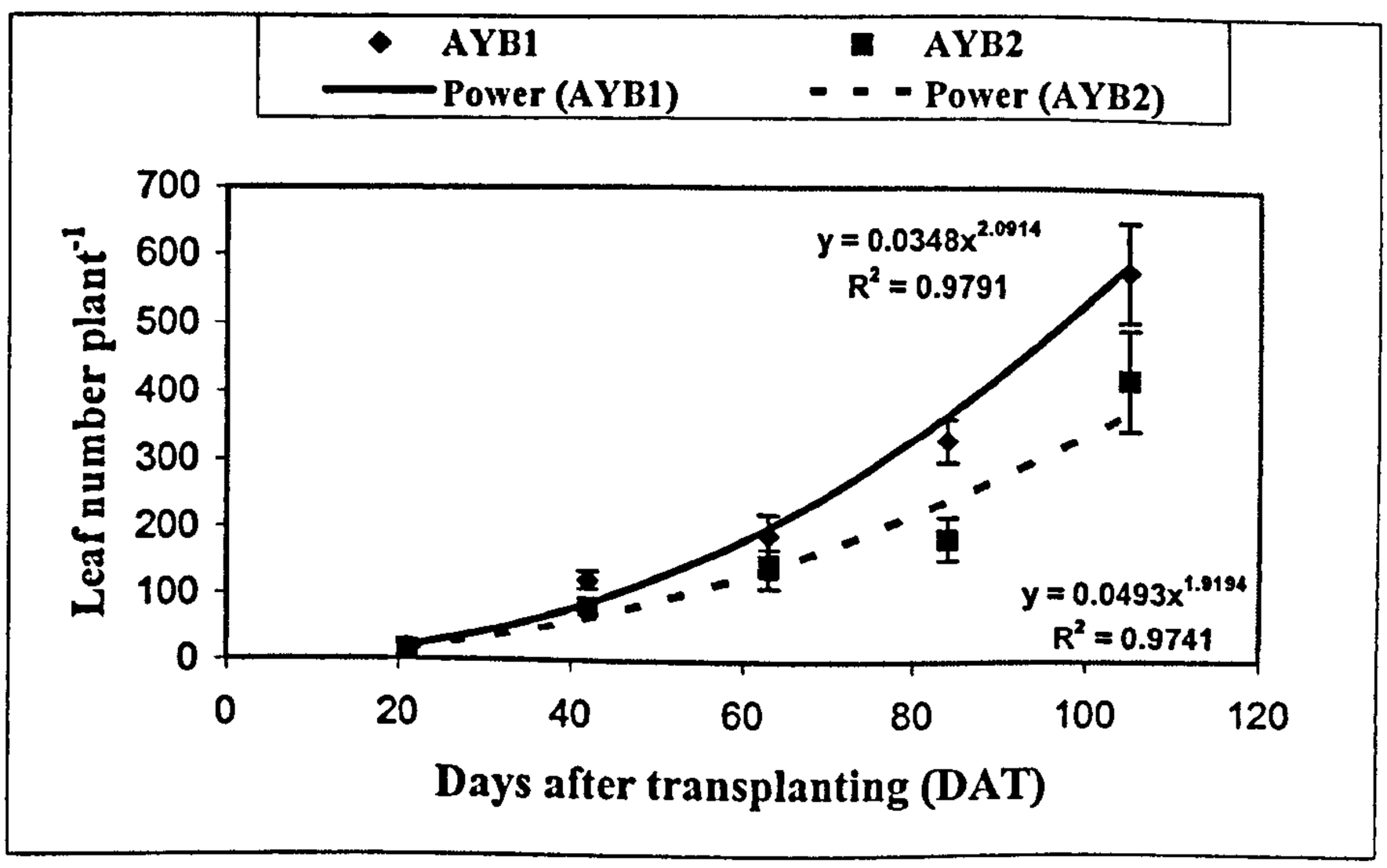

Figure 4.1 Number of leaves of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

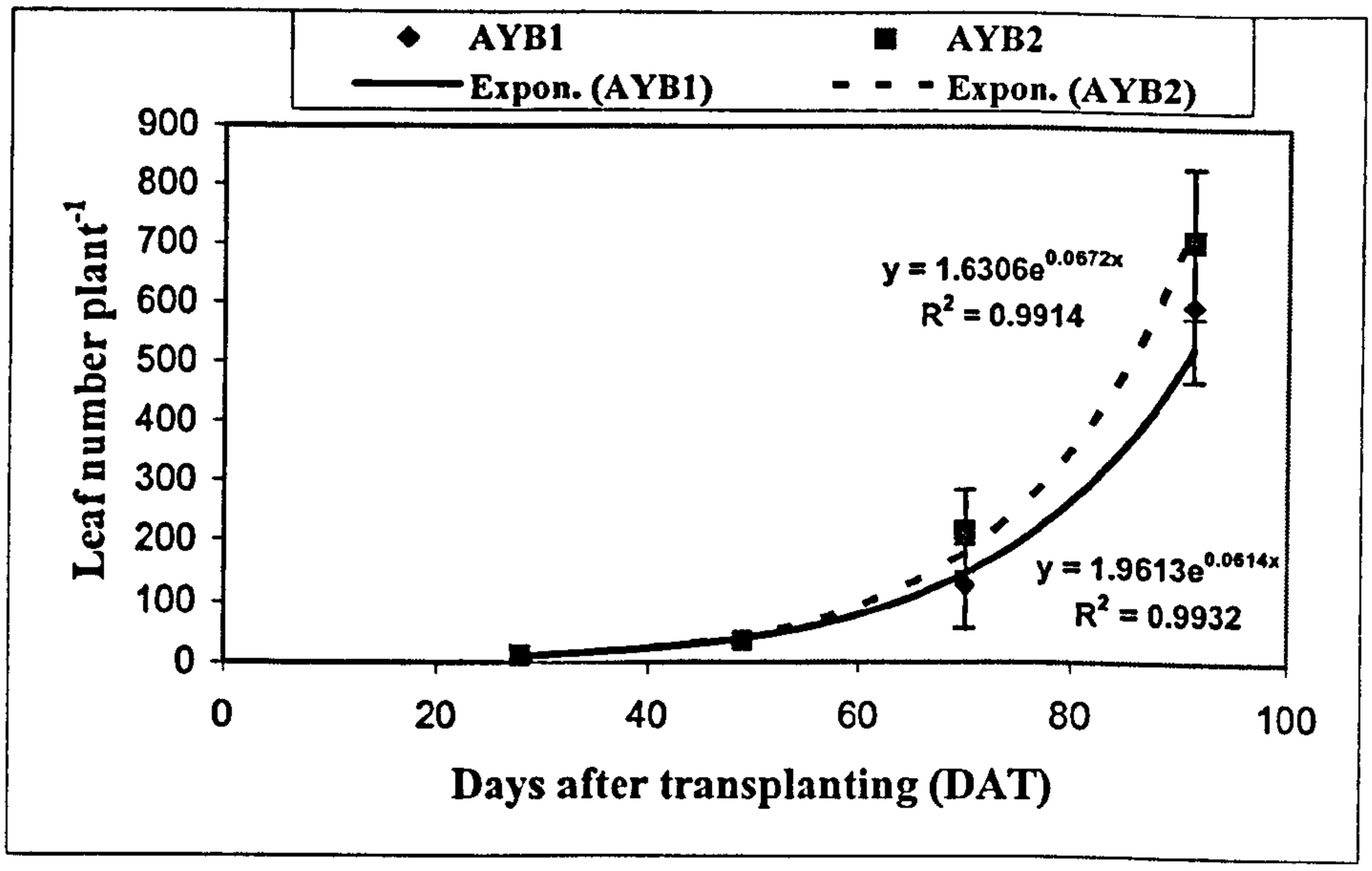

Figure 4.2 Number of leaves of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

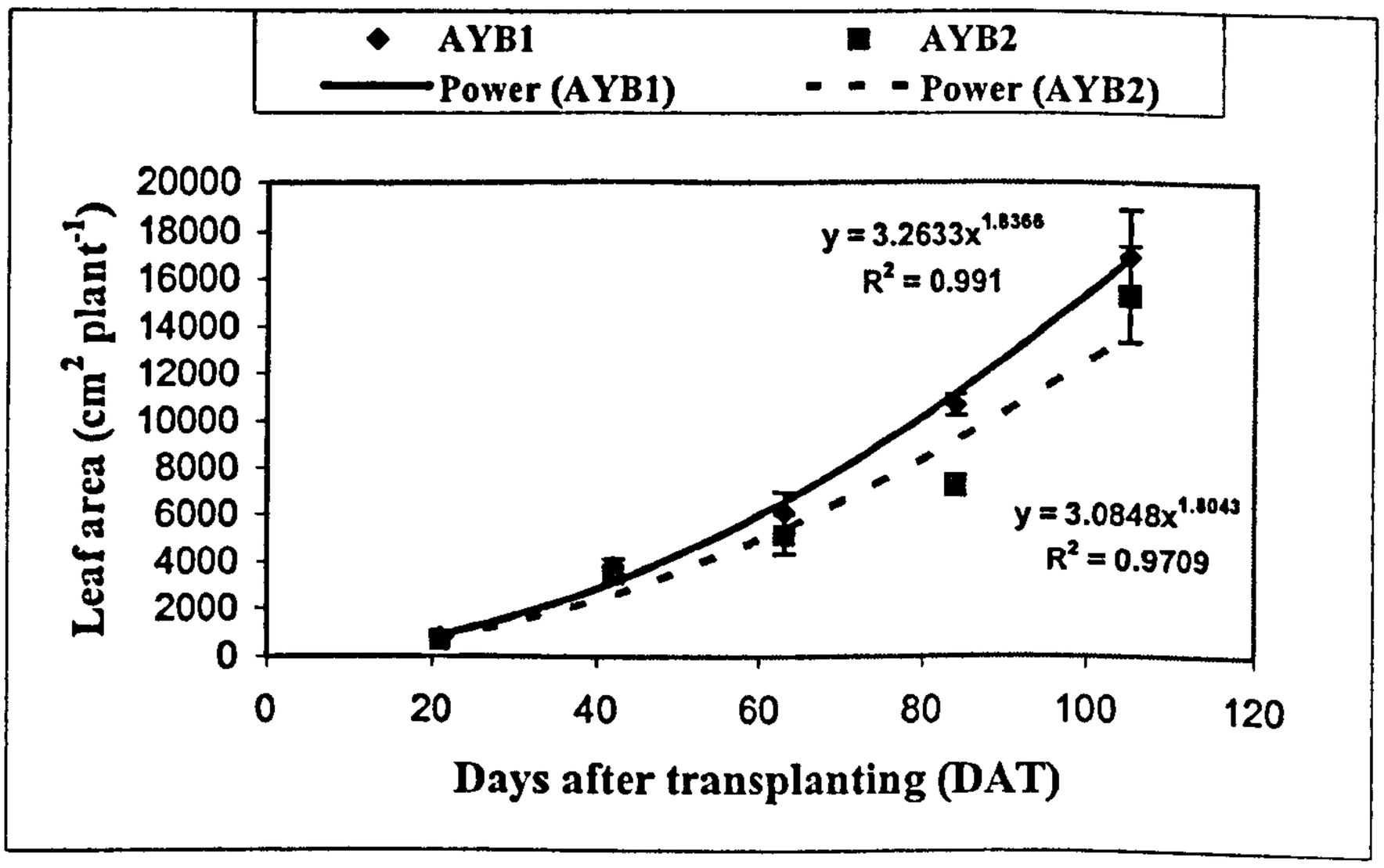

Figure 4.3 Leaf area of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

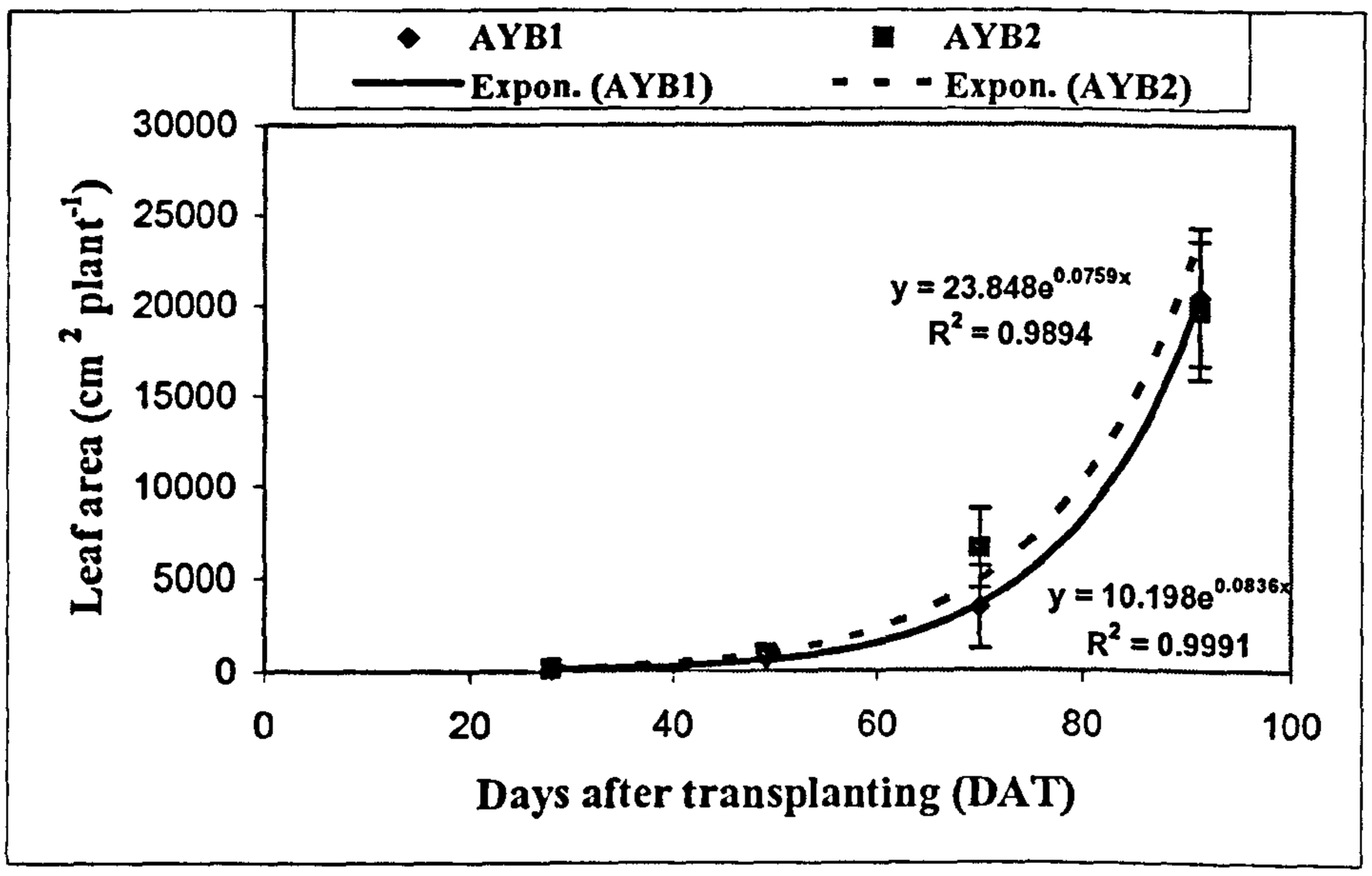

Figure 4.4 Leaf area of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

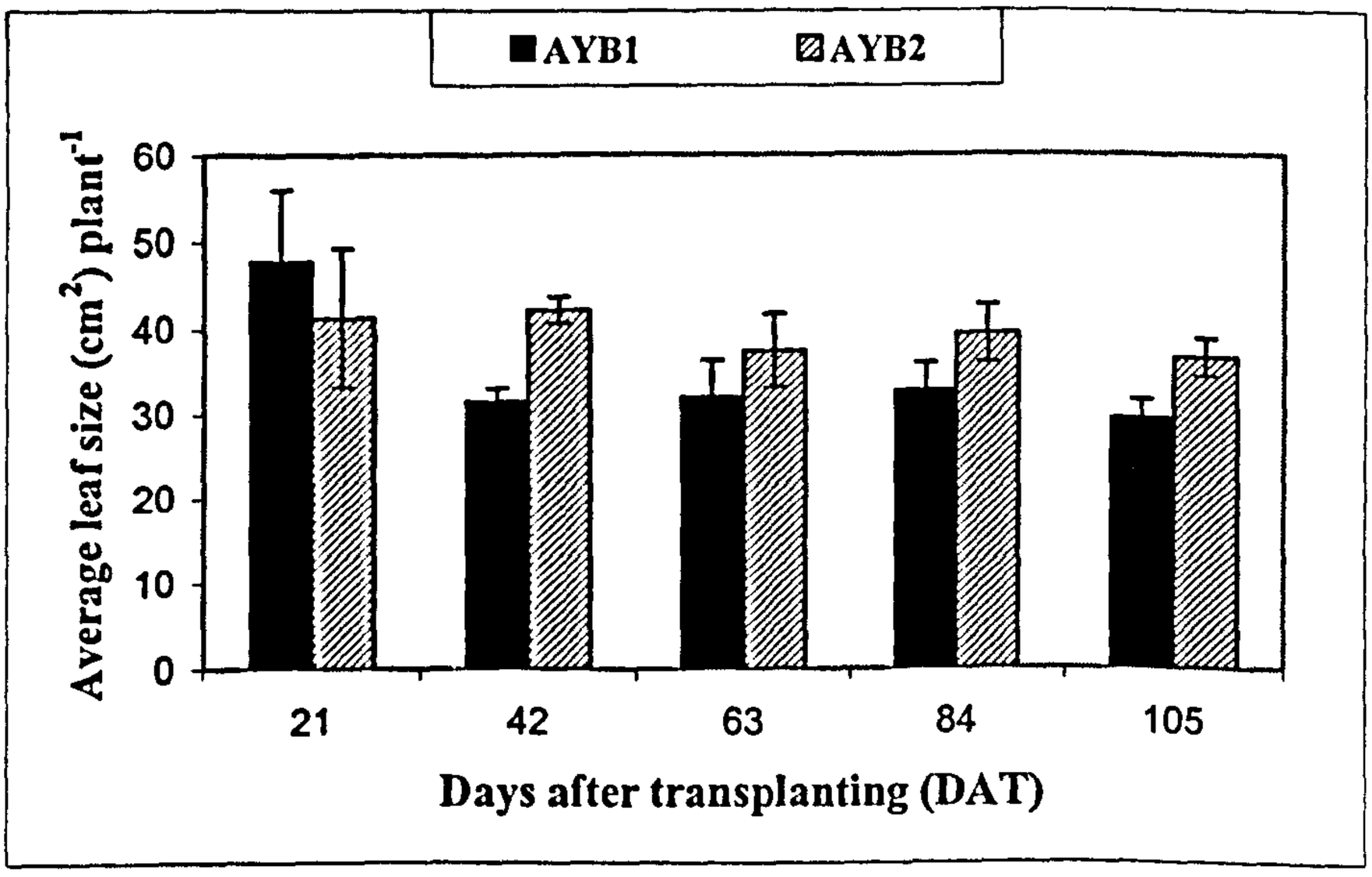

Figure 4.5 Average leaf size of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

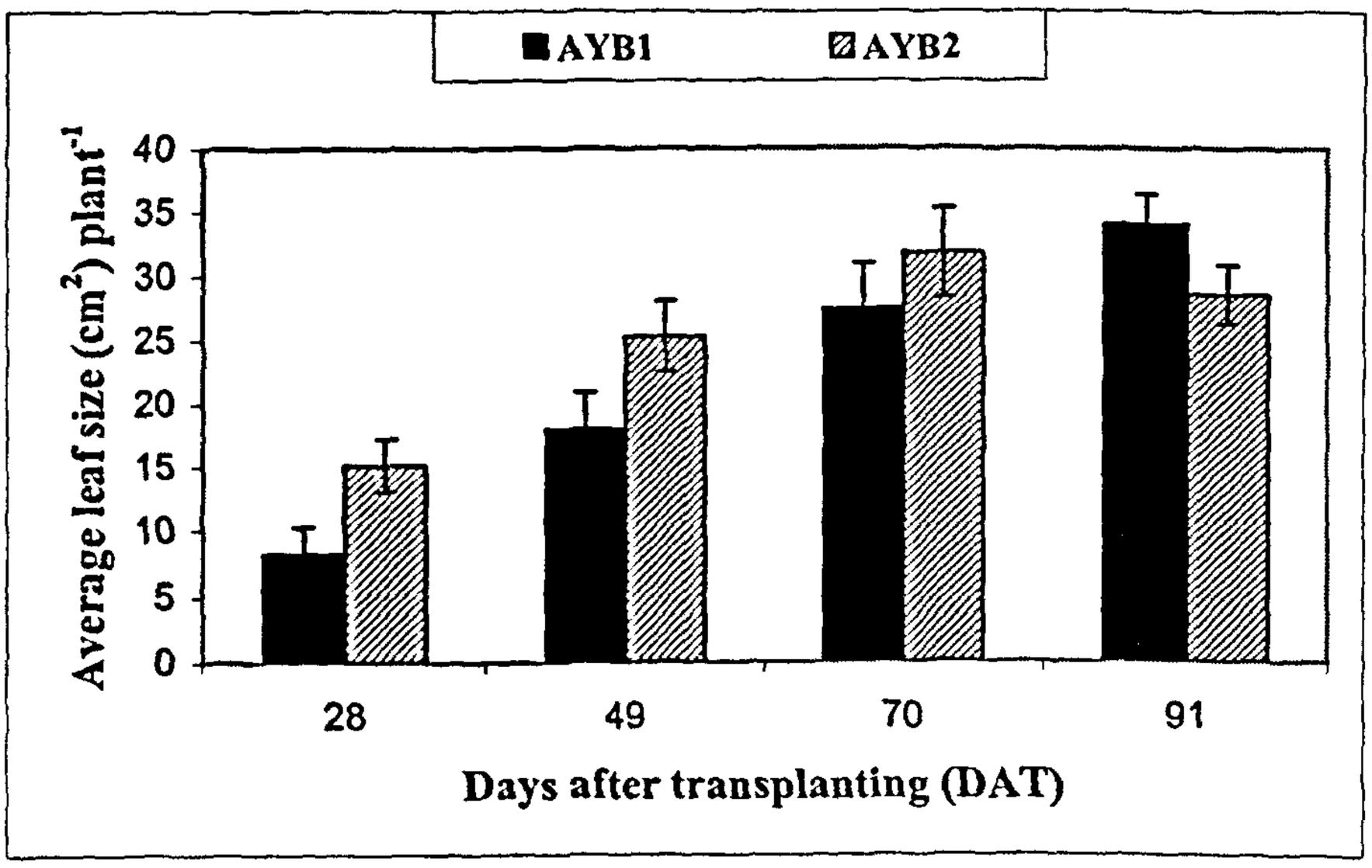

Figure 4.6 Average leaf size of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

4.2.2.1.2 Stem growth

Stem cross sectional area did not vary between the landraces in both experiments, although AYB1 was more vigorous in Experiment 1 and AYB2 grew better in Experiment 2 (Figures 4.7 and 4.8).

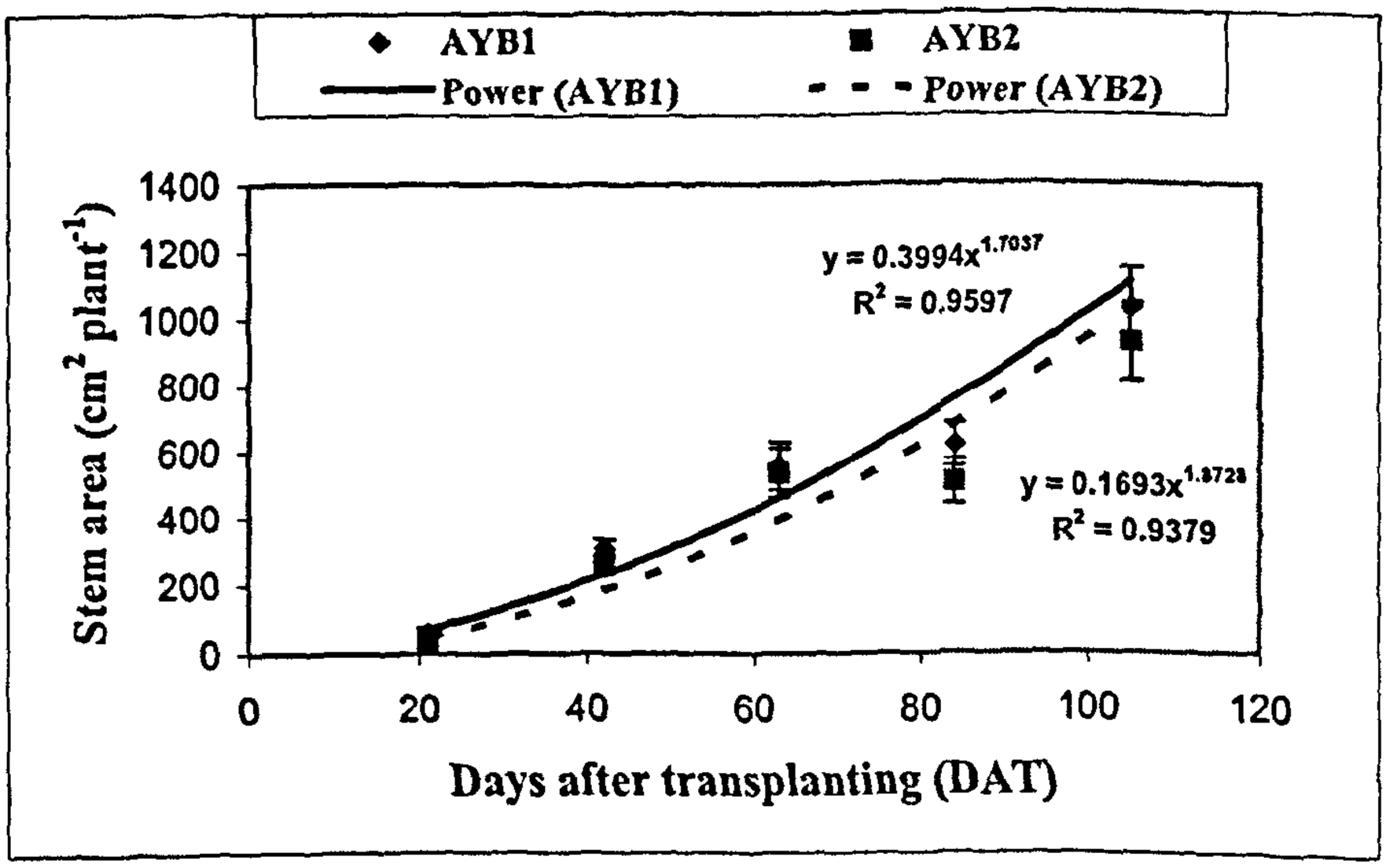

Figure 4.7 Stem growth of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

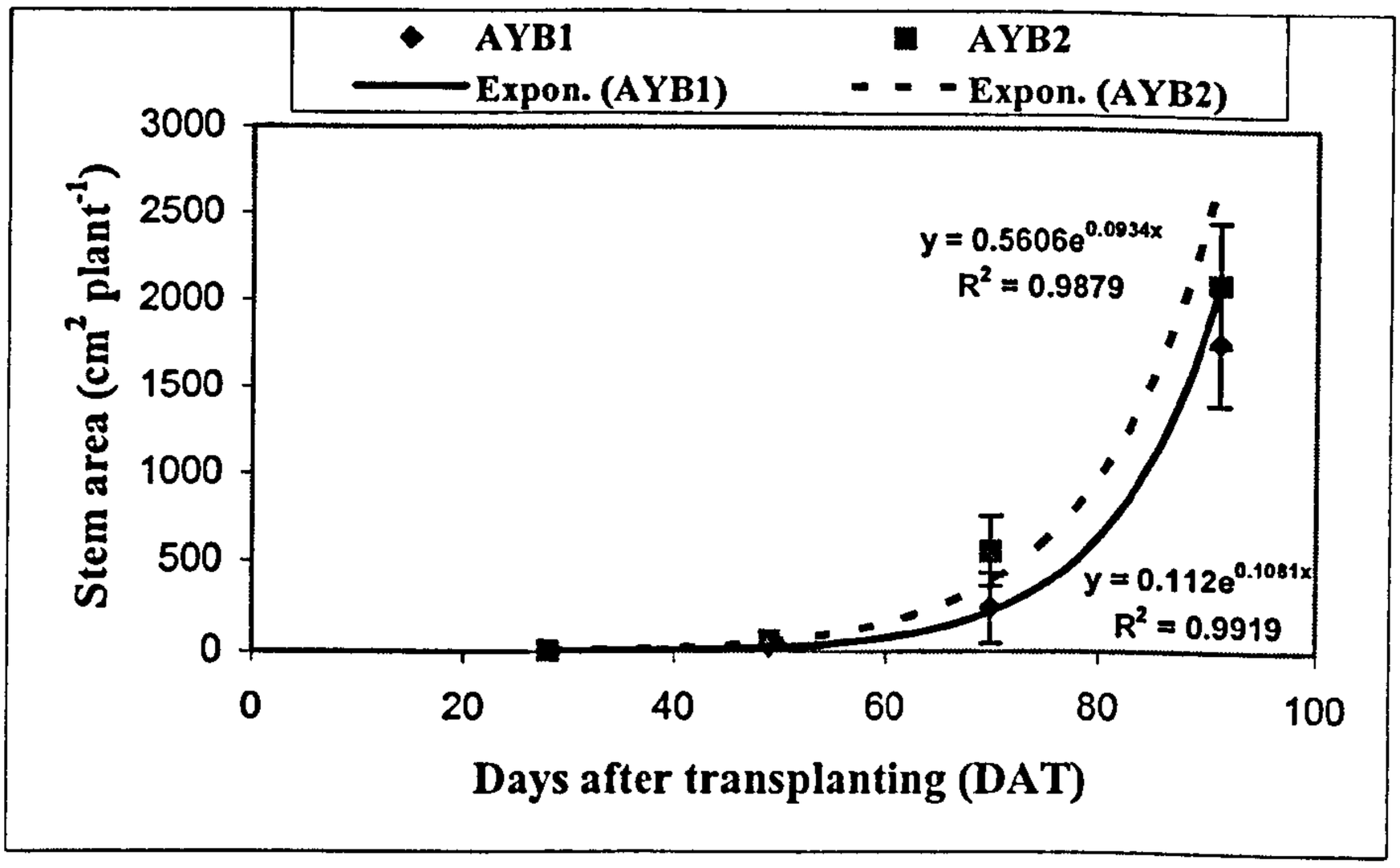

Figure 4.8 Stem growth of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

4.2.2.1.3 Total shoot growth

In Experiment 1, overall shoot growth, as indicated by total green area, showed a superiority of AYB1 over AYB2 although the difference was only significant ($F_{(1,4)}$ = 57.50, p<0.01) at 84 DAT (Figure 4.9). In Experiment 2, the difference between the 2 landraces was not significant at all stages of growth although, AYB2 performed better than AYB1 (Figure 4.10). However, AYB1 shoot growth exceeded AYB2 slightly at 105 DAT. The vegetative growth was indeterminate with both landraces continuing to grow upwards and sideways forming an entangled mesh (Plate 4.9). The largest shoot green area reached by each landrace was 22250 cm^2 (AYB1) and 21852 cm^2 (AYB1) in Experiment 2.

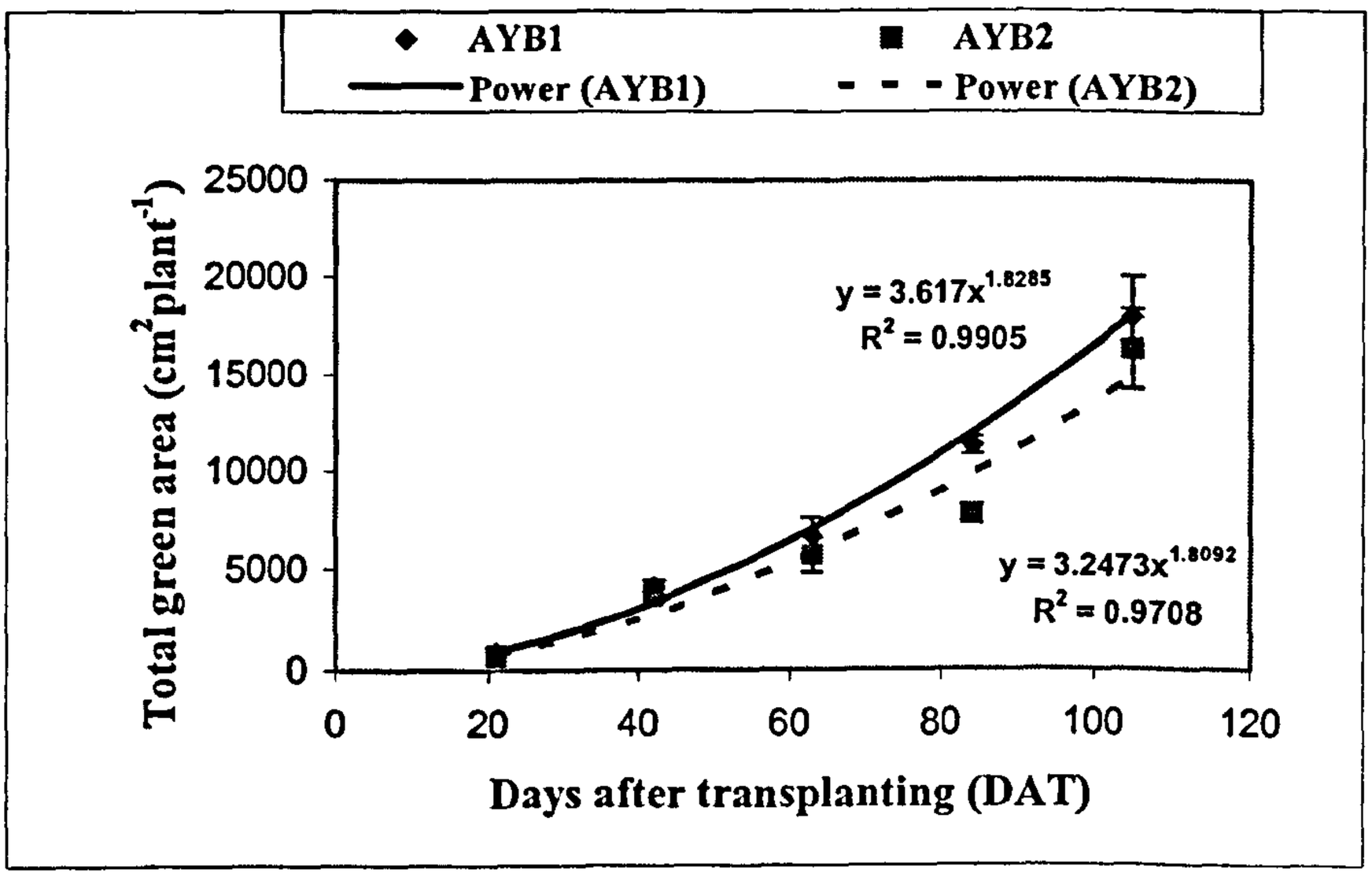

Figure 4.9 Total green area of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

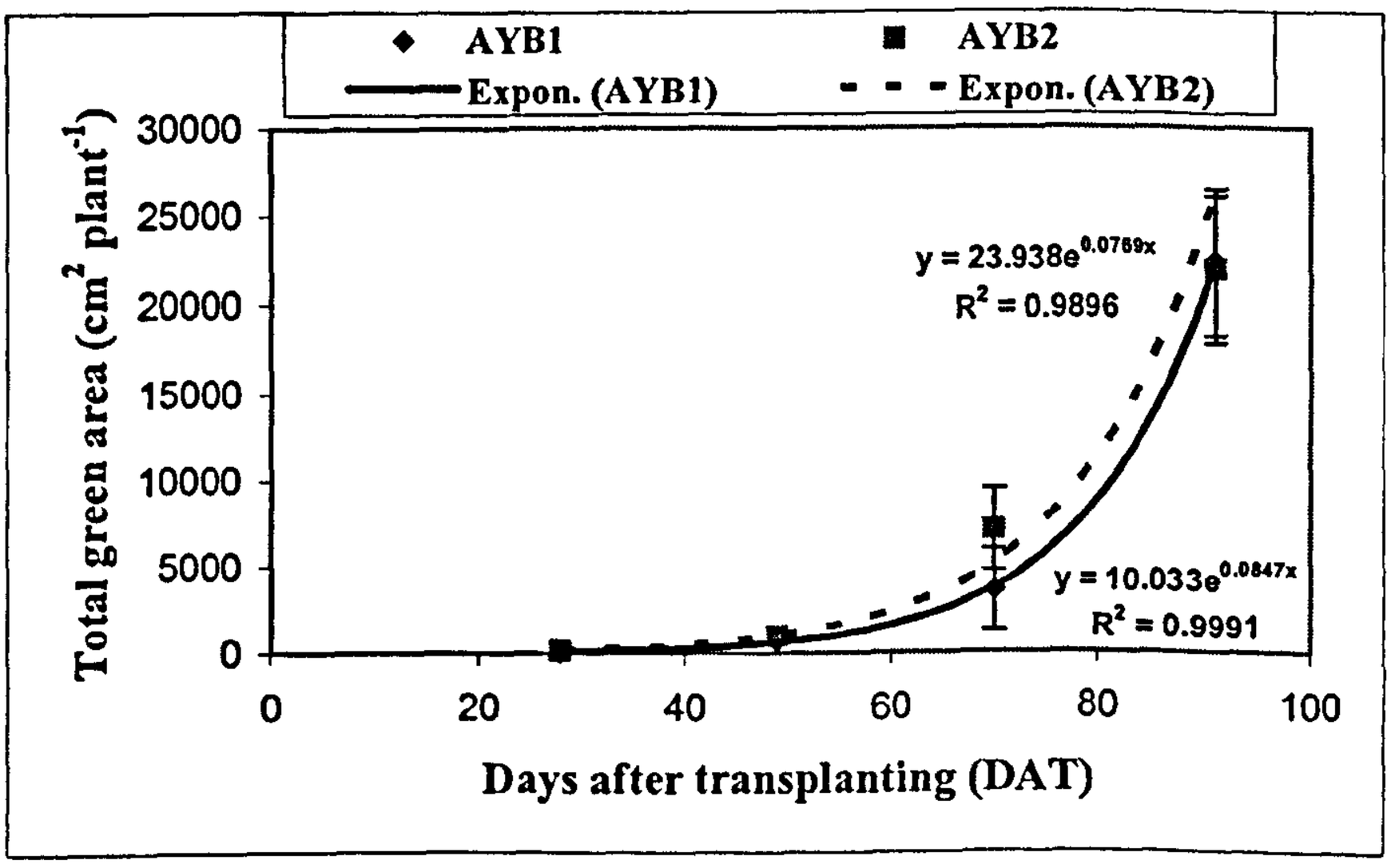

Figure 4.10 Total green area of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

4.2.2.2 Dry matter accumulation

Dry matter accumulation for all of the parameters differed with landrace depending on the growth environment. In the pot experiment, AYB1 performed better than AYB2 in all parameters whereas AYB2 performed better than AYB1 when grown directly in the soil.

4.2.2.2.1 Leaf dry matter

Leaf dry matter accumulation in Experiment 1 was slightly higher in AYB1 than AYB2 with the only significant ($F_{(1,4)}$ = 15.21, p<0.05) difference between the landraces occurring at 84 DAT (Figure 4.11). In Experiment 2, differences between the landraces were not significant (Figure 4.12).

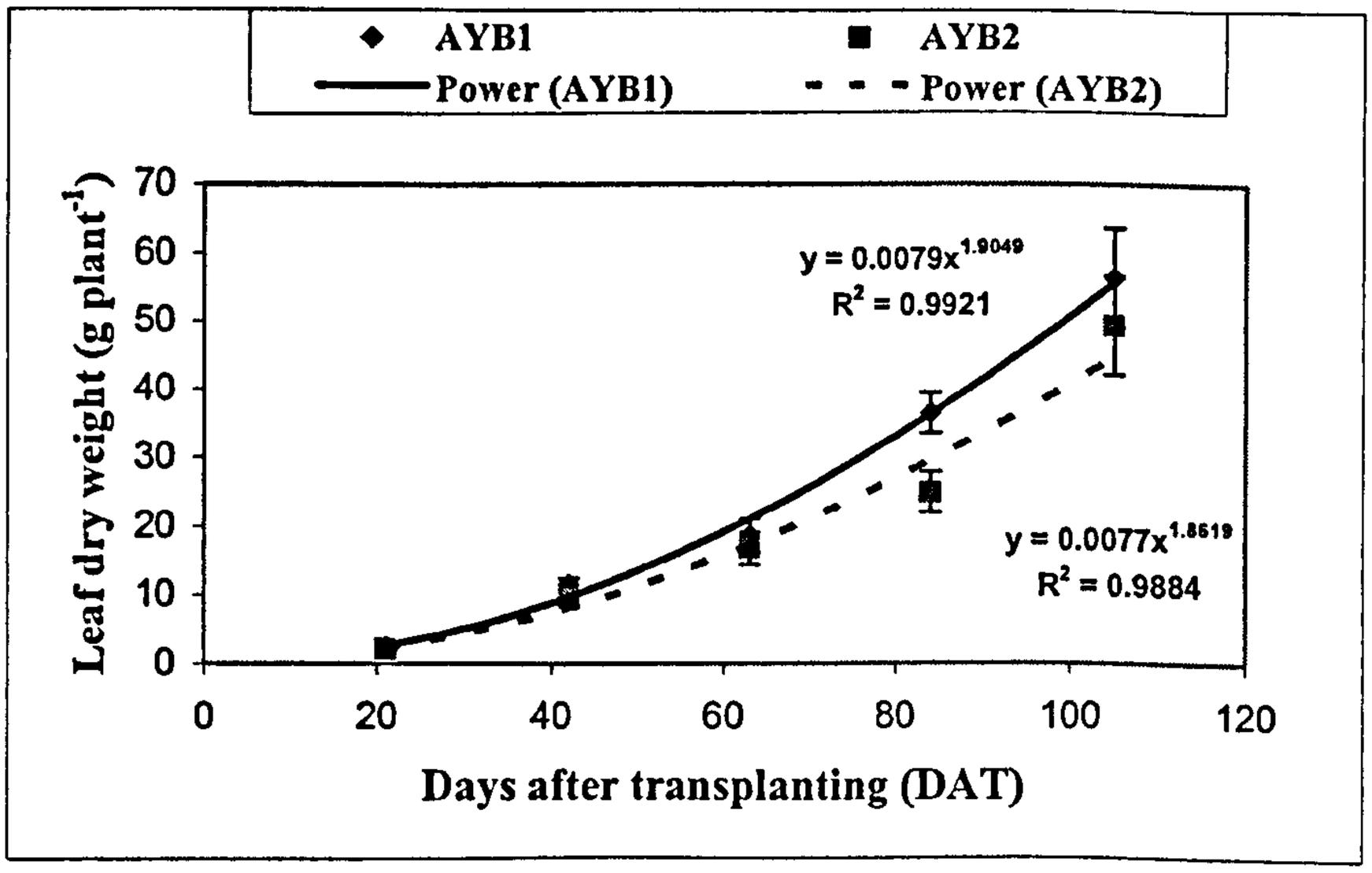

Figure 4.11 Leaf dry matter accumulation of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

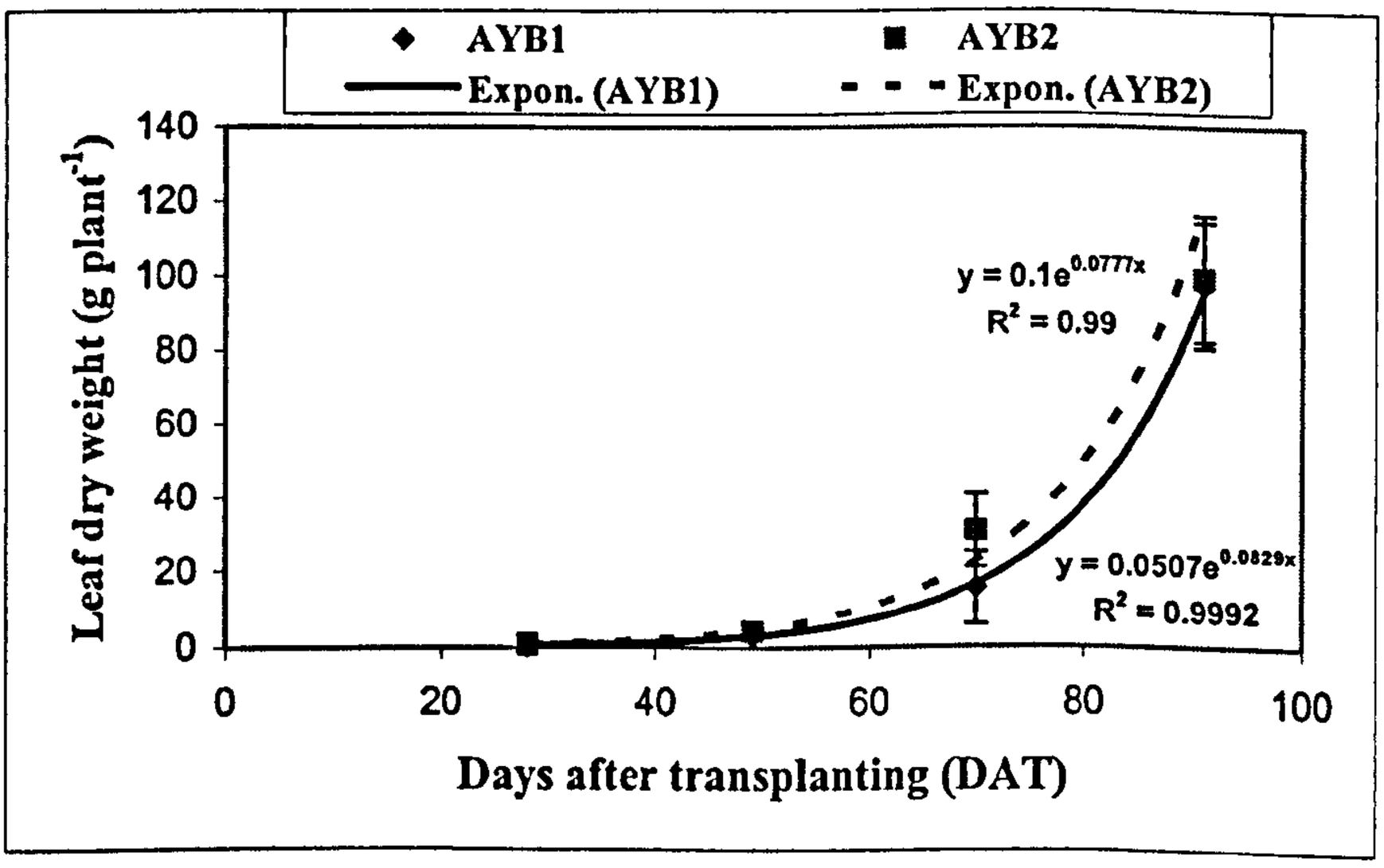

Figure 4.12 Leaf dry matter accumulation of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

4.2.2.2.2 Stem dry matter

Stem dry matter accumulation followed the same trend as leaf dry weight in both experiments, with AYB1 accumulating more dry matter in Experiment 1, while AYB2 was more responsive than AYB1 in Experiment 2 (Figures 4.13 and 4.14). However, the difference between the landraces in both experiments was not significant.

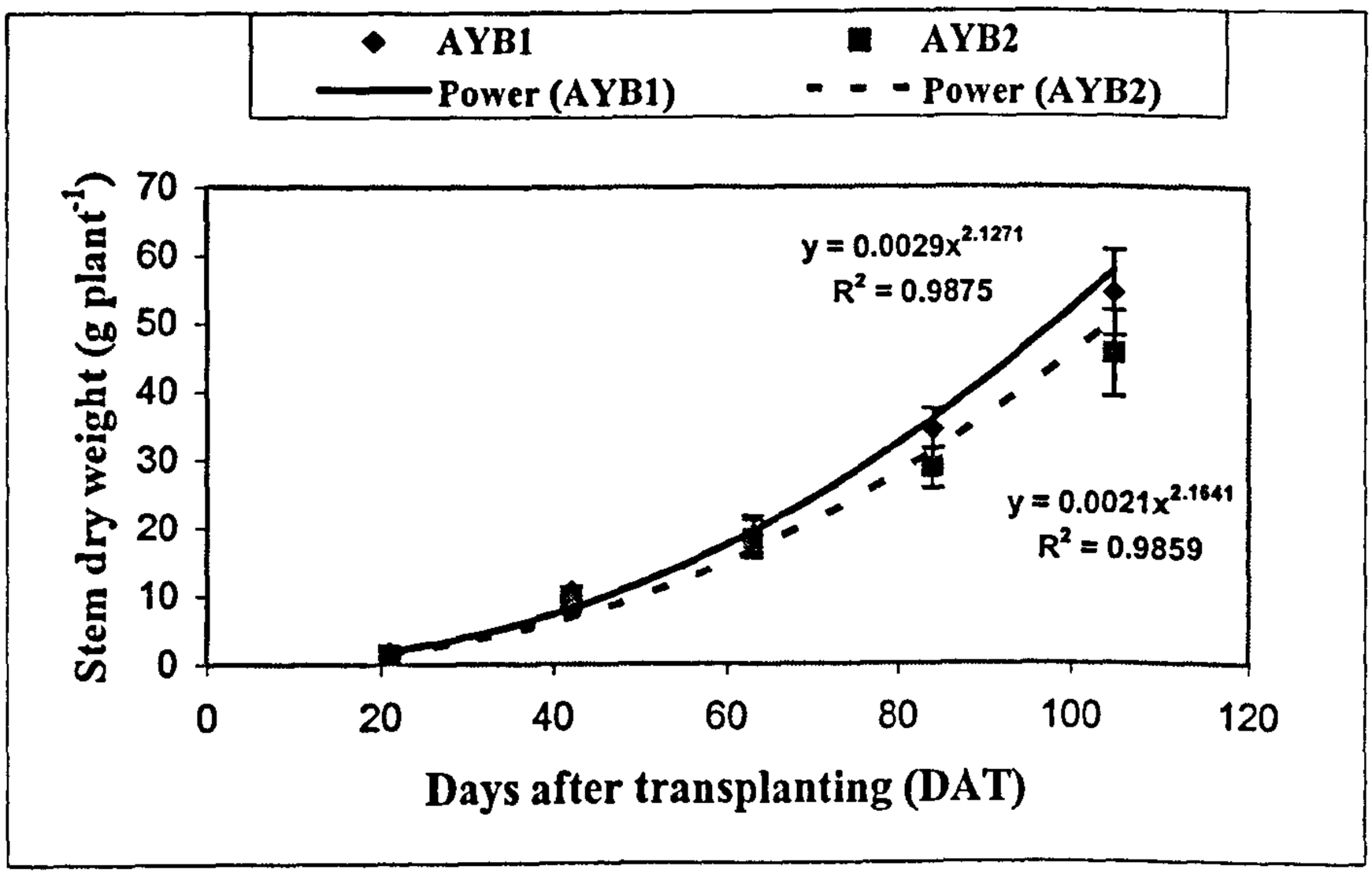

Figure 4.13 Stem dry matter accumulation of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

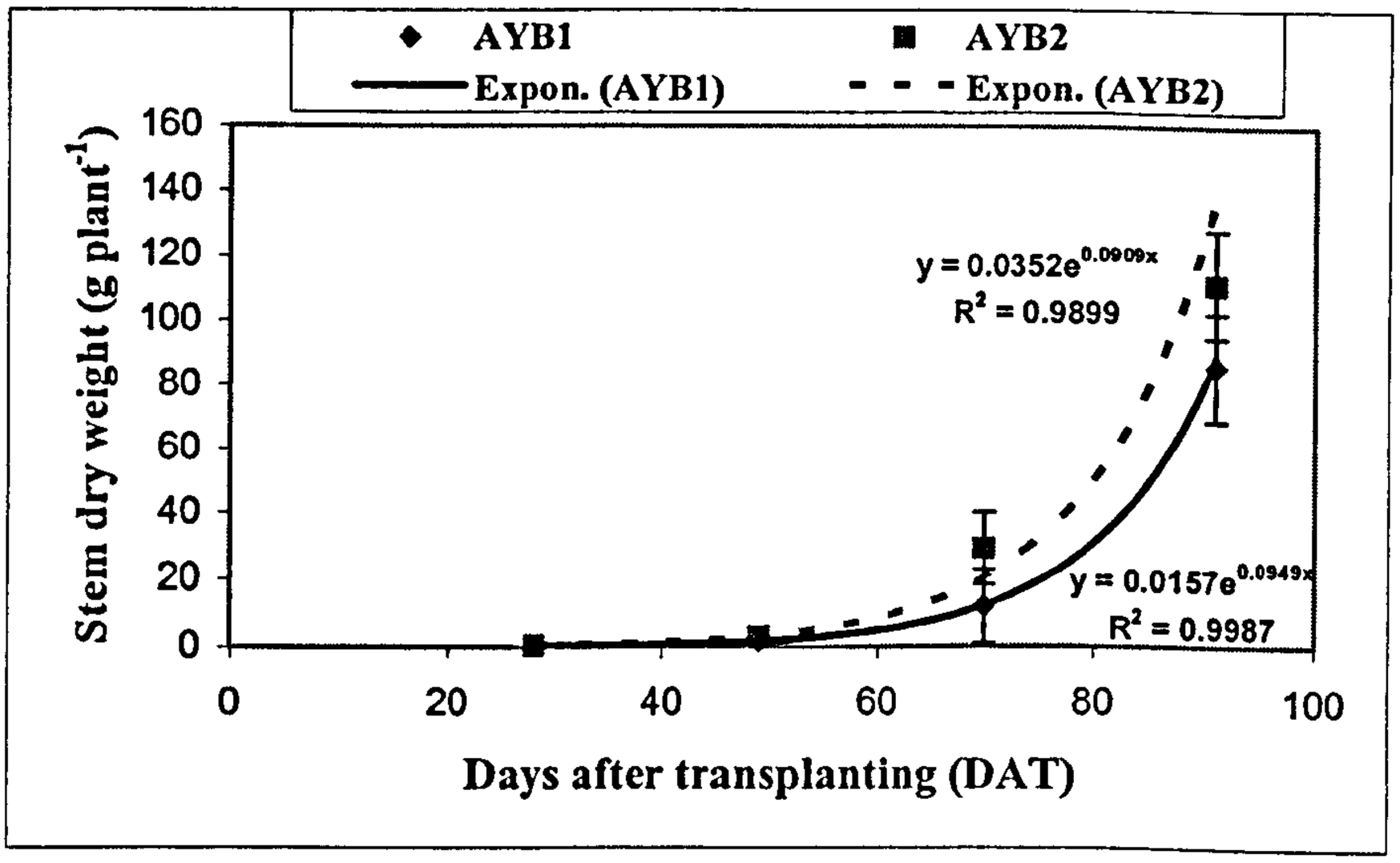

Figure 4.14 Stem dry matter accumulation of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

4.2.2.2.3 Total shoot dry matter

AYB1 appeared to possess higher shoot dry matter than AYB2 in Experiment 1 at all stages of growth. However, differences were only significant at 84 DAT ($F_{(1,4)}$ = 12.52, p<0.05) (Figure 4.15). Differences between the landraces in Experiment 2 were not significant (Figure 4.16). Growth of the 2 landraces was better in the glasshouse soil than in pots. Daily dry matter accumulation was higher in Experiment 2 with an average of 2.89 g d^{-1} (AYB1) and 3.31 g d^{-1} (AYB2) compared to 0.63 g d^{-1} (AYB1) and 0.53 g d^{-1} (AYB2) in Experiment 1 (Table 4.1). Between 70 DAT and 91 DAT, dry matter accumulation increased dramatically exceeding 7 g d^{-1} for both landraces compared to <1 g d^{-1} recorded at the same harvest interval in Experiment 1 (Table 4.1). Plant growth, expressed as crop growth rate (weight of dry matter gained per area per day), ranged from 0.32-11.53 g m^2 d^{-1} for AYB1 and 0.42-11.46 g m^2 d^{-1} for AYB2 (Table 4.1). Overall, AYB1 achieved an average crop growth rate of 4.52 g m^2 d^{-1} while AYB2 achieved 5.17 g m^2 d^{-1} (Table 4.1). Relative growth rate (dry matter accumulated per unit of weight per day) was also high in Experiment 2. In Experiment 1, AYB1 experienced a continued reduction in the rate of dry matter accumulation, while AYB2 slowed down but increased up in the fourth harvest interval (i.e. 84 DAT-105 DAT) (Table 4.2).

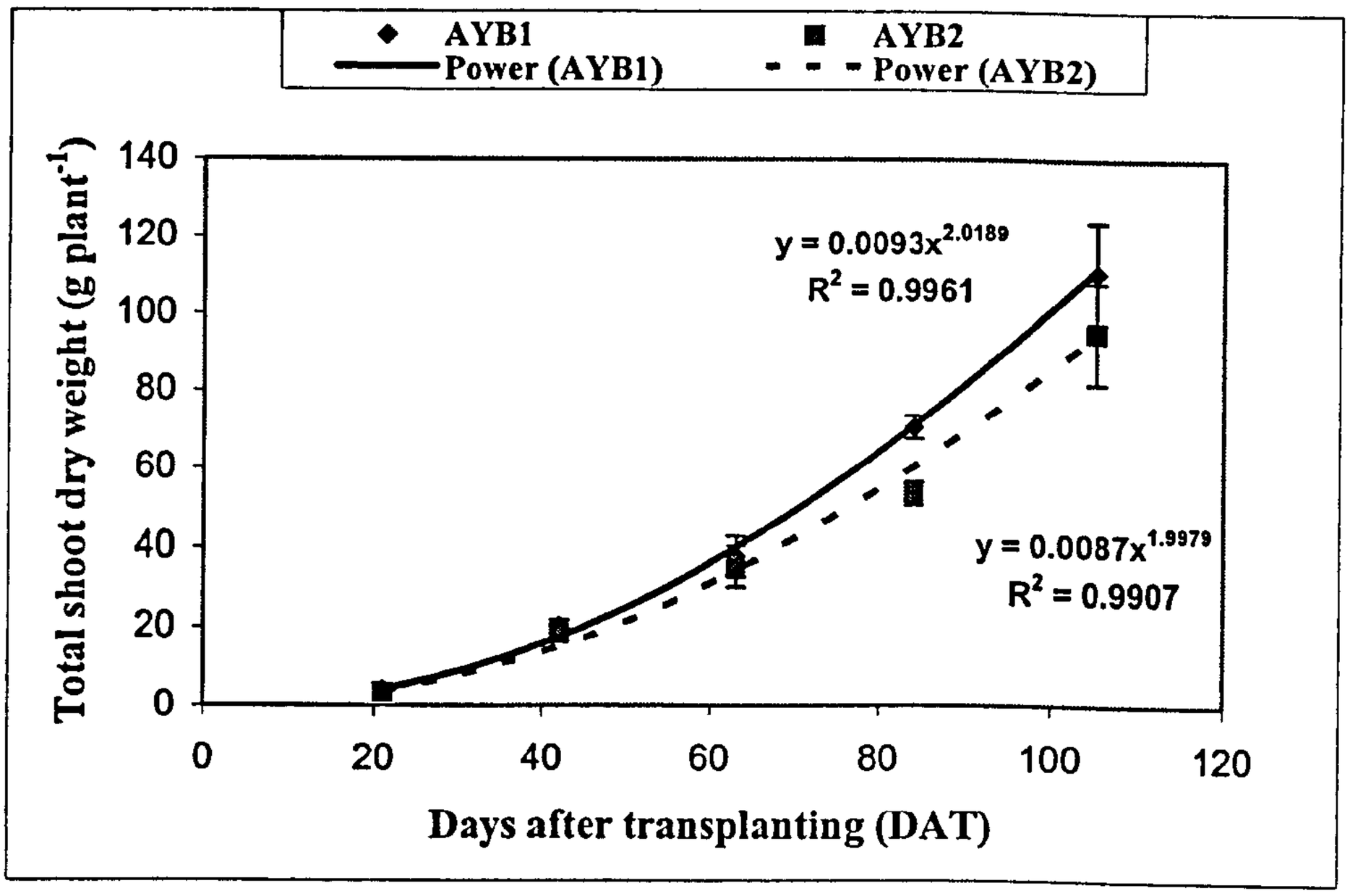

Figure 4.15 Total shoot dry matter of plants of AYB1 and AYB2 grown in pots for up to 105 days. Bar = 2 *s.e.d.* (n=3) (Experiment 1).

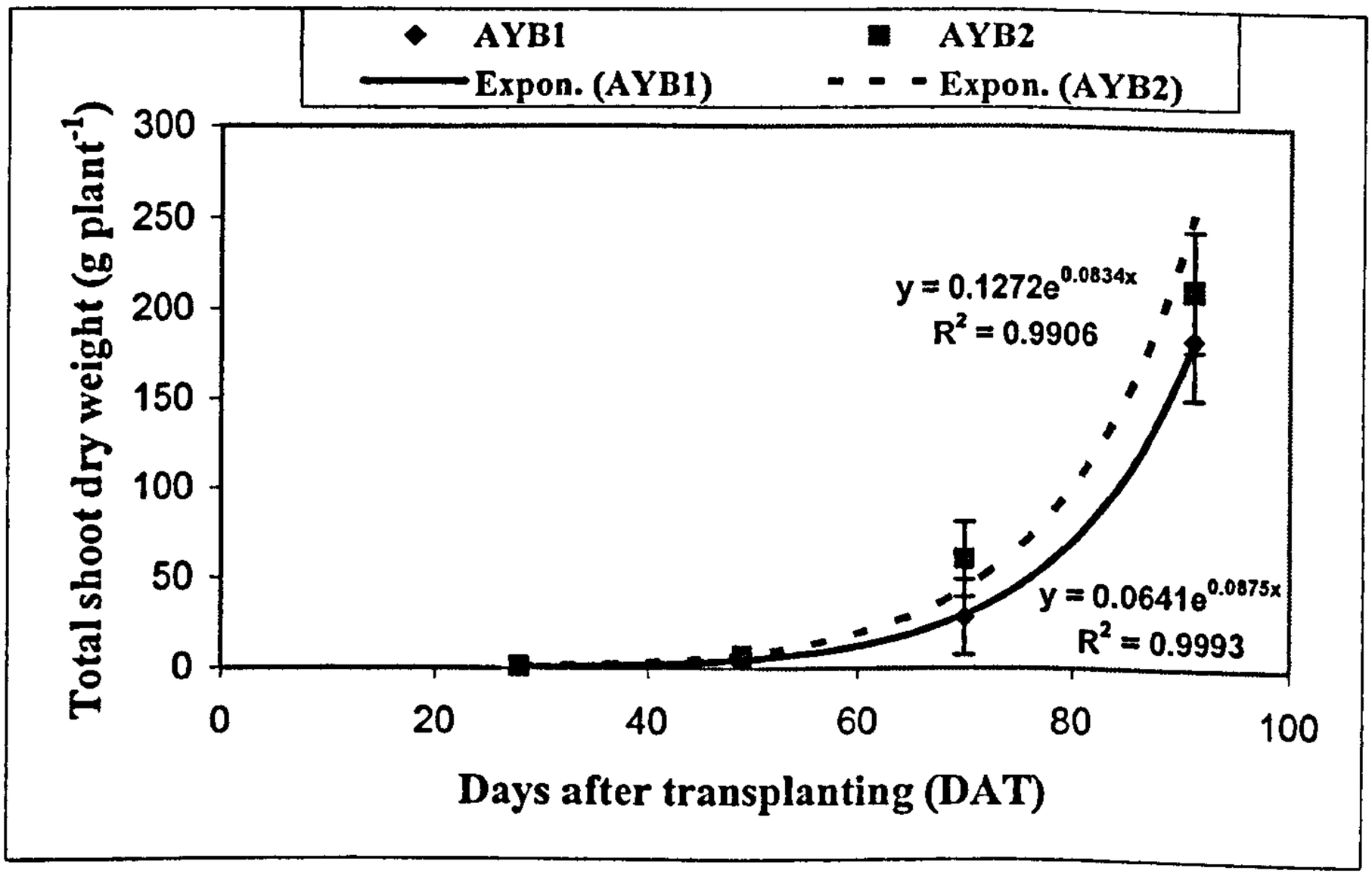

Figure 4.16 Total shoot dry matter of plants of AYB1 and AYB2 grown in soil for up to 91 days. Bar = 2 *s.e.d.* (n=5) (Experiment 2).

Table 4.1 Absolute growth rate (shoot dry matter accumulation) in g plant^{-1} d^{-1} of AYB1 and AYB2 landraces at different harvest intervals.

Harvest interval	Experiment 1 (n=3)		*Experiment 2 (n=5)	
	AYB1	AYB2	AYB1	AYB2
1	0.43	0.36	0.21 (0.32)	0.27 (0.42)
2	0.40	0.45	1.09 (1.70)	2.53 (3.95)
3	0.73	0.49	7.38 (11.53)	7.14 (11.16)
4	0.96	0.80	N/A	N/A
Mean	0.63	0.53	2.89 (4.52)	3.31 (5.17)

N/A = not available because only 4 harvests were carried out

* Number in parenthesis denotes crop growth rate (expressed as g m^2 d^{-1})

Table 4.2 Relative growth rate (shoot dry matter accumulation) in g g^{-1} d^{-1} of AYB1 and AYB2 landraces at different harvest intervals.

Harvest interval	Experiment 1 (n=3)		Experiment 2 (n=5)	
	AYB1	AYB2	AYB1	AYB2
1	0.033	0.035	0.041 (0.064)	0.035 (0.055)
2	0.013	0.014	0.035 (0.055)	0.045 (0.070)
3	0.013	0.009	0.039 (0.061)	0.026 (0.041)
4	0.009	0.012	N/A	N/A
Mean	0.017	0.018	0.038 (0.059)	0.035 (0.055)

N/A = not available because only 4 harvests were carried out

* Number in parenthesis denotes relative crop growth rate (expressed as g m^2 d^{-1})

4.2.3 Discussion

4.2.3.1 Vegetative growth

Growth of the two AYB landraces was indeterminate. Apparent differences between the two landraces were not significant. However, their growth differed with conditions under which they were maintained. AYB1 grew better than AYB2 in pots whereas AYB2 was better in the glasshouse soil. The better growth of plants in the soil than in pots was due possibly to the more extensive root system in the soil as well as adequate nutrients furnished by the fertilizer incorporated at planting. The vigorous vegetative growth of the two landraces resulted in heights of more than 4 m which contradicts the maximum height of 2 m mentioned by Tindall (1983), but agrees with the National Academy of Sciences (1979) report that the plant is a vigorous vine that climbs and twines to heights over 3 m.

Growth was very fast with the plants reaching unmanageable heights after 90 d particularly when planted in the soil. This fast growth agrees with observations in Nigeria, where AYB was one of the four legumes (including local varieties of *Vigna ungiculata, Phaseolus lanatus* and *Phaseolus vulgaris*) selected as the best legume for ground cover, since it achieves 90% ground cover within 6 to 10 weeks after planting (Obiagwu, 1997). This heavy vegetative growth may work positively for tuber yield, although no information is available on the relationship between vegetative and tuber growth. Evidence from other tuber crops, such as yam bean, suggests the importance of building up an adequate photosynthetic apparatus for later enlargement of tubers. Early growth in yam bean under long days of the tropics is characterised by growth with formation of many vine-like shoots that usually result in high tuber and seed yields (Sorensen *et al.*, 1993). A short growth cycle due to late sowing led to reduction in plant growth of yam bean (*Pachyrizus ahipa*) and ultimately resulted in root and tuber yield reductions of 57 and 20% respectively (Leidi *et al.*, 2004). Delay in planting, which effectively shortens the period of vegetative growth, also resulted in reduced seed yield of AYB (Okpara and Omaliko, 1997). This reaffirms the importance of an extended vegetative growth phase in the yield components of AYB and other root tuber crops.

4.2.3.2 Dry matter accumulation

The mean relative growth rates of AYB1 (0.017 and 0.038 g g^{-1} d^{-1}) and AYB2 (0.018 and 0.035 g g^{-1} d^{-1}) recorded in both experiments, although in the range of growth rates of other legumes, do not compare favourably. Short season soybean (*Glycine max* L.) grown under different row spacing and planting density had growth rates in the range of 0.132-<0.01 g g^{-1} d^{-1} (Herbert and Litchfield, 1984). Dry matter accumulation was slow early in the growth of plants in the soil compared with those in pots, but increased dramatically towards the last stage. This could be attributed to a shock that plants experienced when they were transplanted from optimal nursery conditions to glasshouse soil. As growth progressed and the plants became established with a free root run in contrast to those in the pots, dry matter accumulation improved remarkably from 0.21-7.38 g $plant^{-1}$ d^{-1} (AYB1) and 0.27-7.14 g $plant^{-1}$ d^{-1} (AYB2). It is likely that, once established, plants grown in the soil experience minimum physiological stress as roots were able to exploit a large reservoir of water, nutrients and even air.

When converted to average crop growth rate, AYB1 grew by 4.52 g m^2 d^{-1} and AYB2 by 5.17 g m^2 d^{-1}, considerably less than the growth rates of other tropical legumes, such as cowpea. Maximum crop growth rate of cowpea grown in experimental plots in the savanna zone has been reported to be 15 g m^2 d^{-1} (Summerfield *et al.*, 1983). Crop growth rates will vary with the genotype and prevailing environmental conditions, such as solar radiation. For example, Ntare and Willians (1993) recorded average crop growth rates of 0.72, 1.35 and 1.61 g m^2 d^{-1} for 3 cultivars of cowpea grown during the cool period (characterised by night temperature between 15 and 20°C) of the Sahelian zone of West Africa. It is clear from observations with cowpea that both genotype and the prevailing environment played a significant role. Although growth rates achieved in this study were low compared to those recorded for cowpea in the savanna zone, it is conceivable that AYB would achieve equally high growth rates if grown in a conducive environment.

Although evidence for variation in the growth of two AYB landraces has been presented in this study, it has not been possible to identify the impact that variation will have on reproductive growth as the plants in the two growth trials did not flower. The reproductive potential shown by stock plants grown under cooler conditions indicates a possibility of thermal control of flowering in AYB that needs further investigation. During the summer, day temperature in the glasshouse sometimes exceed 30 °C and this could have affected the flowering process in same way as what happened when stock plants were kept in growth room with similar temperature. Continued vegetative growth, dry matter accumulation and the production of flowers and seeds by stock plants indicates that AYB plants have an indeterminate growth habit. It was not possible to determine the onset of formation of the subterranean tubers. An integrated study of the phenology and physiology of the species is required to characterise the developmental pattern that will provide guidelines for production.

CHAPTER 5

PROPAGATION BY STEM CUTTINGS

5.1 Introduction

A simple rapid technique is required for the induction of a vigorous root system on stem cuttings that will shorten the growth cycle, provide high transplant survival and result in vigorous plants (Vesperinas, 1998). Rooting of cuttings for easy to root species is usually used as a rapid method of asexual/clonal propagation to achieve genetically identical plants. Adventitious root formation is a developmental process comprised of a sequence of histological events each marked by stages with different requirements (Hartmann *et al.*, 1997). A number of factors, including auxin concentration, propagule position on the mother plant and genotype, play an important role in the formation of adventitious roots. In this study some of the above-mentioned factors are evaluated and the following hypotheses tested:

- Formation of adventitious roots is genotypically controlled.
- Adventitious root formation requires exogenous auxin application.
- Efficacy of auxin in inducing root formation vary with concentration.
- Physiological status (leaf size and position of propagule on mother plant) of the propagule influences adventitious root formation.

5.2 Effect of auxin concentration on rooting of AYB stem cuttings

Auxins play an important role in the promotion of rooting in many plant species although they have also been found to be inhibitory to rooting if applied in high concentrations (Henry *et al.*, 1992; Chee, 1995; Goh *et al.*, 1995; Garrido *et al.*, 1996; Nikam and Shitole, 1997; Cameron *et al.*, 2001). Aminah *et al.* (1995) observed that auxin (IBA) significantly increased the rate of root emergence in leafy stem cuttings of *Shorea leprosula,* but that higher doses (more than 20 μg per cutting) resulted in less rooting success. Dunn *et al.* (1996) also found differences in root formation and growth between cuttings of *Pistacia chinensis* treated with different concentrations of IBA. However, Almehdi *et al.*, (2002) tried IBA potassium salt at 0, 40.0 and 80 g l^{-1} on pistachio (*Pistachio* spp.) and found no

significant difference between these concentrations on percentage of rooted cuttings. Mesén *et al.*, (1997) recorded a reduction in percentage rooting of *Cordia alliodora* when concentration of IBA was elevated within the range of 0-1.6 % w/v of methanol.

Shoot initiation and development is sometimes adversely affected even though there has been adequate root formation due to inhibition of bud development by the application of synthetic auxins at high concentrations (Hartmann *et al.*, 1997). Therefore, the aim of this experiment was to test the hypothesis that auxin promotes root formation and different auxin concentrations vary in the effectiveness to induce root formation and growth in AYB. The following were the guiding objectives for this investigation:

- determine the requirement for exogenous auxin application for rooting of AYB cuttings.
- identify the suitable auxin concentration for rooting AYB cuttings.

5.2.1 Materials and methods

Two experiments were carried out to determine the suitable auxin concentration for rooting of AYB stem cuttings using aqueous potassium salts of IBA at the following concentrations based on literature cited above:

Experiment 1. 0. (control), 5.0 and 20 g l^{-1}

Experiment 2. 0.0 (control), 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100 g l^{-1}

Treatments in both experiments were applied by dipping the stem basal end of nodal cuttings to a depth of 2 cm in the auxin solution for 60 s before the cuttings were placed in the rooting medium as described in Chapter 3, Section 3.5.2. In Experiment 1, there were 15 cuttings per treatment per propagator (described in Chapter 3, Section 3.5.2) per landrace while in Experiment 2 there were 5 cuttings per treatment per propagator per landrace. In Experiment 1, number of rooted cuttings, number of roots per cutting and root length were recorded at the end of the experiment (21 d), while in Experiment 2, in addition to parameters measured in the first experiment, number of rooted stem cuttings was recorded every 3 days to determine the rate of root formation over 15 d. Cuttings rooted in Experiment 1 were potted in compost to check their subsequent survival.

5.2.2 Results

5.2.2.1 Root formation

Adventitious roots originated from different positions on the cuttings depending on the treatment imposed. Cuttings that did not receive any auxin (control) formed roots at the base from the wound/callus, whereas cuttings treated with auxin produced roots higher up the stem and not necessarily from the basal wound (Plate 5.1). It was evident that auxin application caused damage to the stem base with the loss of bark and no roots arising from that region (Plate 5.1).

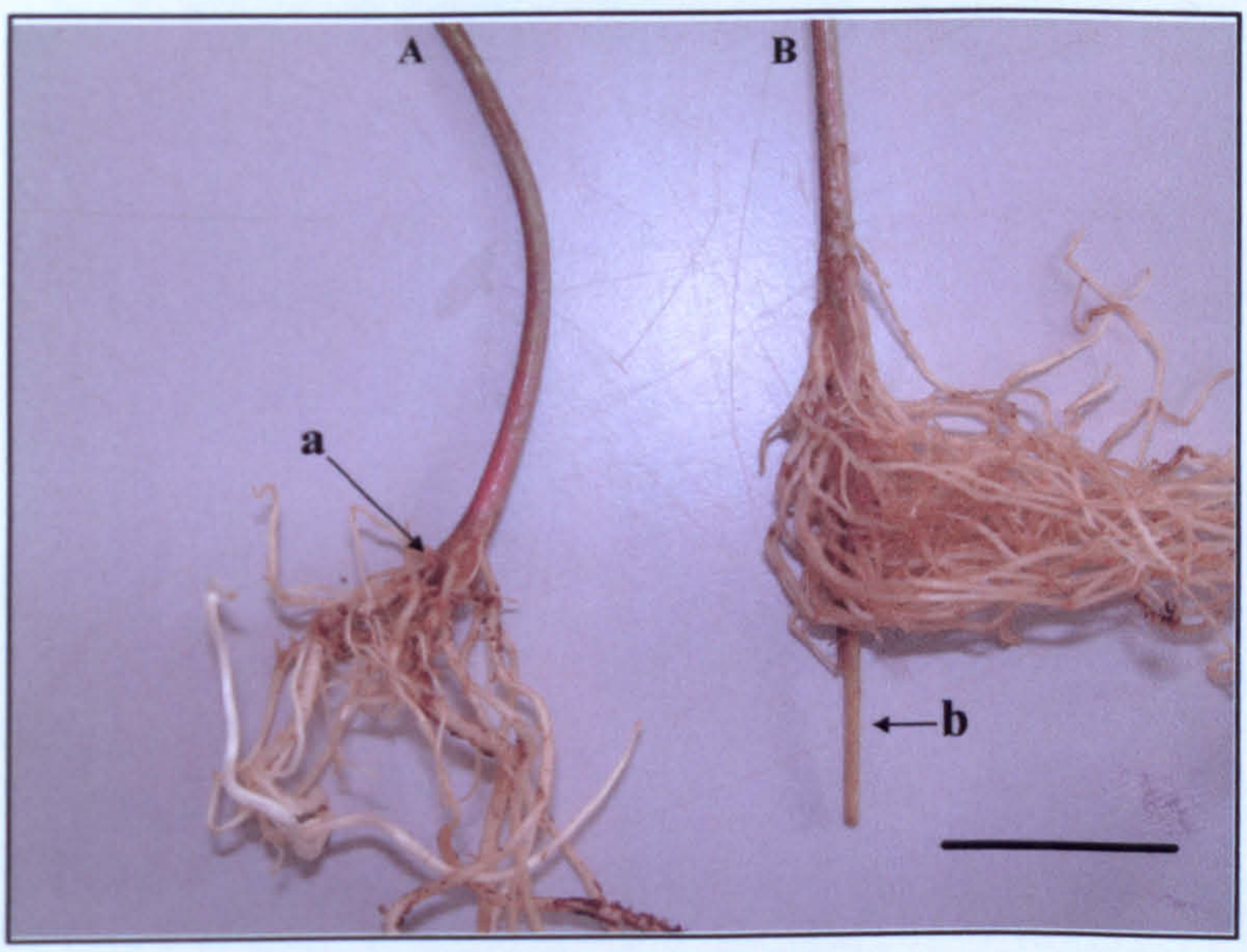

Plate 5.1 Adventitious roots formed on cuttings (A) without auxin roots forming mostly on wounded portion (a) and (B) with auxin, note, no roots formed on the damaged portion (b) of the stem basal end of the cutting that was in contact with auxin. Bar = 15 mm.

In Experiment 2, although there was a trend towards faster root formation on cuttings treated with auxin, it was not possible to make a clear comparison between auxin concentrations due to high mortality of cuttings, particularly those receiving the greater auxin doses. Nevertheless, AYB landraces were shown to be amenable to propagation by cuttings as all of the live cuttings had rooted in the second week of both experiments. In Experiment 1, all of the control cuttings and most of the cuttings in the 5 g l^{-1} auxin treatment formed roots. However, root formation in the 20 g l^{-1} treatment was much reduced (Table 5.1). The auxin concentration did not seem to influence the rate of root formation, since cuttings that were treated with

auxin did not root any faster than the controls. Instead, auxin adversely affected cuttings as shown by the high number of mortalities. In AYB1, the difference between the proportions of cuttings rooted in week 1 and week 2 were 3.3, 5 and 10 % for the control, 5.0 and 20 g l^{-1} auxin respectively indicating faster rooting in the auxin-free treatment. Mortalities did not occurr in the control cuttings.

In Experiment 2, with the use of more auxin concentrations and the time between observations reduced to 3 days, a trend was evident towards enhanced root formation with increase in auxin concentration but this declined above 2.5 g l^{-1} (Table 5.2). Mortality of cuttings also occurred in the control treatment, although it was more in the auxin treatments. Mortalities were mostly associated with fungal infection and could not be blamed entirely on auxin concentration as the control cuttings experienced more mortalities than some of the auxin treated cuttings. Rooting was not observed at day 3 for either of the landraces in any of the treatments, but by day 6, rooting had occurred in all treatments for both landraces. Rooting of AYB2 was slow with less than 10 rooted cuttings in any treatment at day 6. Although rooting started slowly for AYB1, it had improved markedly by days 9 and 12 for the control treatment while it was relatively slow for AYB2. The proportion of rooted cuttings on the highest auxin treatments (50 and 100 g l^{-1}) was lower than the controls for both landraces (Table 5.2). Overall, best root formation occurred with 0.25 to 5.0 g l^{-1} which had the lowest mortalities. AYB2 showed a higher sensitivity to high auxin treatment than AYB1 in Experiment 2 (Table 5.2) whereas the opposite occurred in Experiment 1 (Table 5.1).

Table 5.1 Effect of auxin concentration on rooting of stem cuttings of AYB1 and AYB2 landraces (Experiment 1).

Auxin Concentration (g l^{-1})	Weekly cumulative number rooted (n=60)				Number of dead cuttings	
	AYB1		AYB2		AYB1	AYB2
	Week1	Week2	Week1	Week2		
0.0	58	60	60	60	0	0
5.0	56	59	52	57	1	7
20.0	13	19	26	33	41	27

Table 5.2 Effect of auxin concentration on rooting of stem cuttings of AYB1 and AYB2 landraces (Experiment 2).

Auxin concentration ($g\ l^{-1}$)	Cumulative number of rooted cuttings (n=25)										Number of dead cuttings after 15 d	
	AYB1					AYB2					AYB1	AYB2
	d 3	d 6	d 9	d 12	d 15	d 3	d 6	d 9	d 12	d 15		
0	0	2	17	21	21	0	3	12	17	19	4	6
0.25	0	5	22	22	22	0	3	23	25	25	3	0
0.50	0	5	20	21	22	0	2	16	21	22	3	2
1.00	0	10	19	19	20	0	3	25	25	25	5	0
2.50	0	13	21	22	22	0	7	24	25	25	3	0
5.00	0	10	22	22	22	0	6	24	24	24	3	1
10.00	0	7	18	20	20	0	6	21	21	21	5	4
25.00	0	6	18	18	22	0	6	20	21	24	3	1
50.00	0	6	16	18	18	0	4	13	13	13	7	12
100.00	0	1	14	18	19	0	0	4	4	5	6	20

5.2.2.2 Root number

Application of auxin to cuttings increased the number of roots formed per cutting in both experiments (Plate 5.1; Tables 5.3 and 5.4). In Experiment 1, there was no significant difference between the number of roots on cuttings treated with the 2 auxin concentrations, but they both differed ($F_{(2,30)}$ = 12.87, p<0.001) from the control (Table 5.3). When more auxin concentrations were assessed in Experiment 2, there was also a highly significant difference ($F_{(8,162)}$ = 40.76, p<0.001) between auxin treatments. The number of roots per cutting seemed to increase with elevation of the auxin concentration, but declined after 25.0 g l^{-1} (Table 5.4). There was no difference between the control and 0.25 to 0.5 g l^{-1} auxin treatments. However, cuttings treated with 1.0 to 50 g l^{-1} auxin had more roots than the control cuttings. The highest root numbers were recorded on cuttings that received 25 g l^{-1} auxin. Differences between the 2 landraces were significant in Experiment 1 ($F_{(1,30)}$ = 4.26, p<0.05) and Experiment 2 ($F_{(1,162)}$ = 15.27, p<0.001) with AYB1 outperforming AYB2 with highs of 94.8 roots compared to 76.3 roots (Experiment 1) and 84.3 to 53.9 roots (Experiment 2).

5.2.2.3 Root length

The effect of auxin on root length seemed to be the opposite of root number although differences were only slightly significant in Experiment 1 ($F_{(2,30)}$ = 3.51, p<0.05) and Experiment 2 ($F_{(8,162)}$ = 2.02, p<0.05) (Tables 5.3 and 5.4). In Experiment 1, cuttings that did not receive any auxin had the longest roots (Table 5.3 and Plate 5.2). In Experiment 2, root length of AYB1 appeared to be enhanced by auxin at 0.25 and 5.0 g l^{-1} and reduced at concentrations >10.0 g l^{-1}. This trend was not apparent with AYB2. Differences in root length between the landraces were not significant, even though AYB1 appeared to produce longer roots (Experiment 1, 148.0 mm and Experiment 2, 146.0 mm) than AYB2 (Experiment 1, 117.0 mm and Experiment 2, 114.6 mm) (Tables 5.3 and 5.4).

Plate 5.2 Effect of auxin concentration on root length (A; long roots with no auxin and B; short roots with auxin treatment). Bar = 20 mm.

Table 5.3 Effect of auxin concentration on mean number of roots per cutting and mean root length of AYB1 and AYB landraces. (n=5) (Experiment 1)

Treatment	**Number of roots cutting^{-1}**		**Root length (mm)**	
	AYB1	AYB2	AYB1	AYB2
Auxin concentration (IBA g l^{-1})				
0.0 (control)	35.2	17.3	148.0	117.5
5.0	94.8	57.0	112.5	99.5
20.0 g l^{-1}	78.8	76.3	92.5	91.0
s.e.d.	16.26		22.22	
d.f.	30		30	
Significance (0.05)				
Landrace	0.048		0.252	
Auxin concentration	<0.001		0.043	
Interaction	0.319		0.653	

Table 5.4 Effect of auxin concentration on mean number of roots per cutting and mean root length of AYB1 and AYB2 landraces. (n=10) (Experiment 2)

Treatment	Number of roots cutting^{-1}		Root length (mm)	
	AYB1	AYB2	AYB1	AYB2
Auxin concentration (IBA g l^{-1})				
0.0 (control)	6.4	7.1	100.2	71.2
0.25	13.4	9.0	146.0	104.0
0.50	11.0	11.1	142.2	97.9
1.00	19.4	17.2	101.5	105.8
2.50	27.2	26.0	127.2	87.2
5.00	38.0	29.0	111.3	114.6
10.00	50.3	36.2	78.0	74.5
25.00	84.3	53.9	78.5	100.5
50.00	64.2	45.4	83.2	88.6
s.e.d.	6.77		24.02	
d.f.	162		162	
Significance (5%)				
Landrace	<0.001		0.088	
Auxin concentration	<0.001		0.047	
Interaction	0.017		0.374	

5.2.2.4 Shoot growth

Shoot height indicated a negative effect of auxin on shoot/bud growth (Plate 5.3, Figures 5.1 and 5.2). In Experiment 1, shoots were significantly ($F_{(2,30)}$ = 54.05, p<0.001) taller (225.5 mm) from cuttings that were not treated with auxin (Figure 5.1). Differences between treatments were also significant ($F_{(2,162)}$ = 3.06, p<0.01) in Experiment 2 with a similar trend to that observed in Experiment 1. In Experiment 2, control cuttings did not grow any better than those treated with auxin at 0.25 to 5.0 g l^{-1}. Differences between the landraces were highly significant ($F_{(1,162)}$ = 23.52, p<0.001) with AYB2 showing more sensitivity to auxin than AYB1 (Figure 5.2).

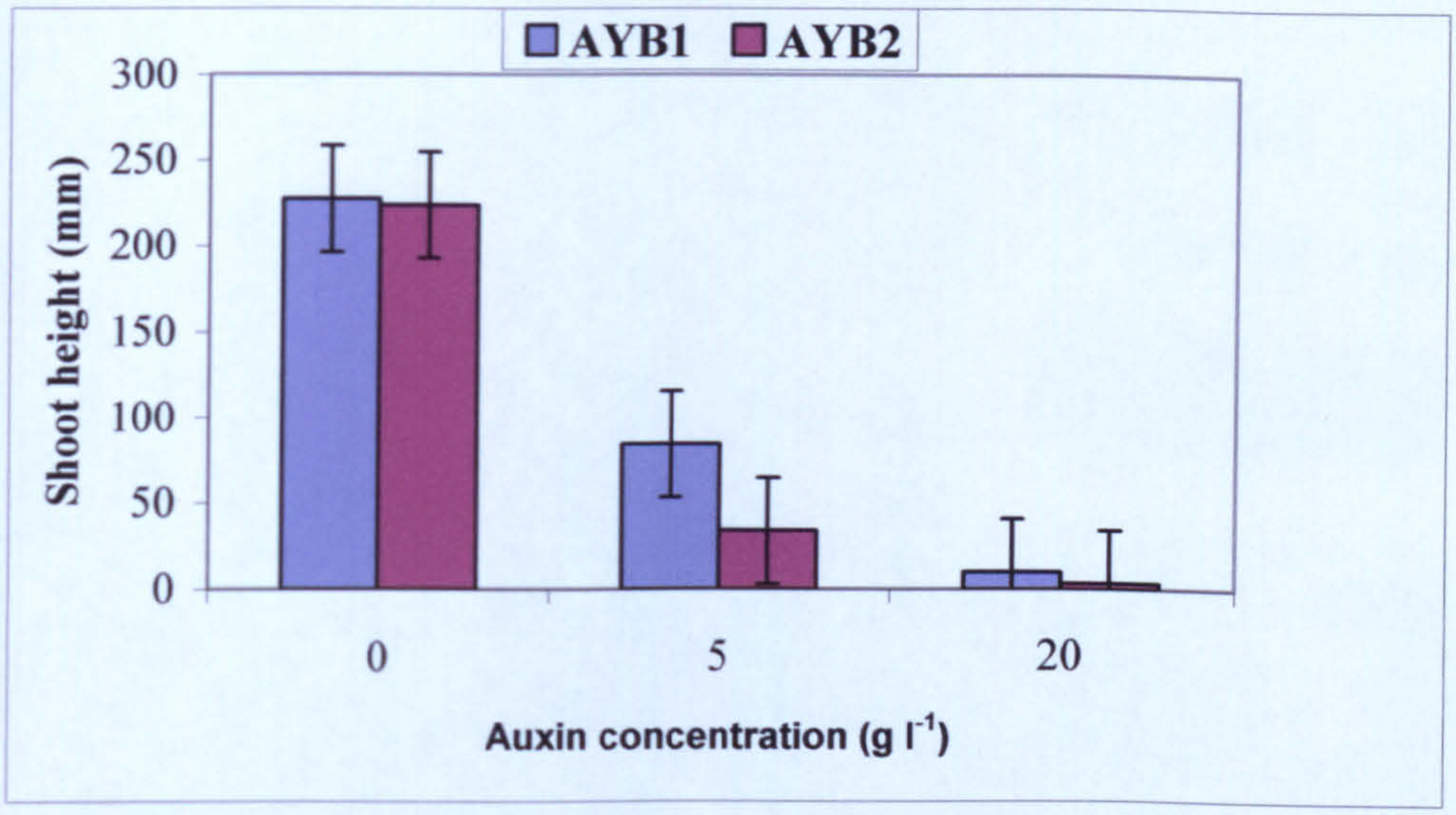

Figure 5.1. Effect of auxin concentration on bud growth (shoot height) of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5) (Experiment 1)

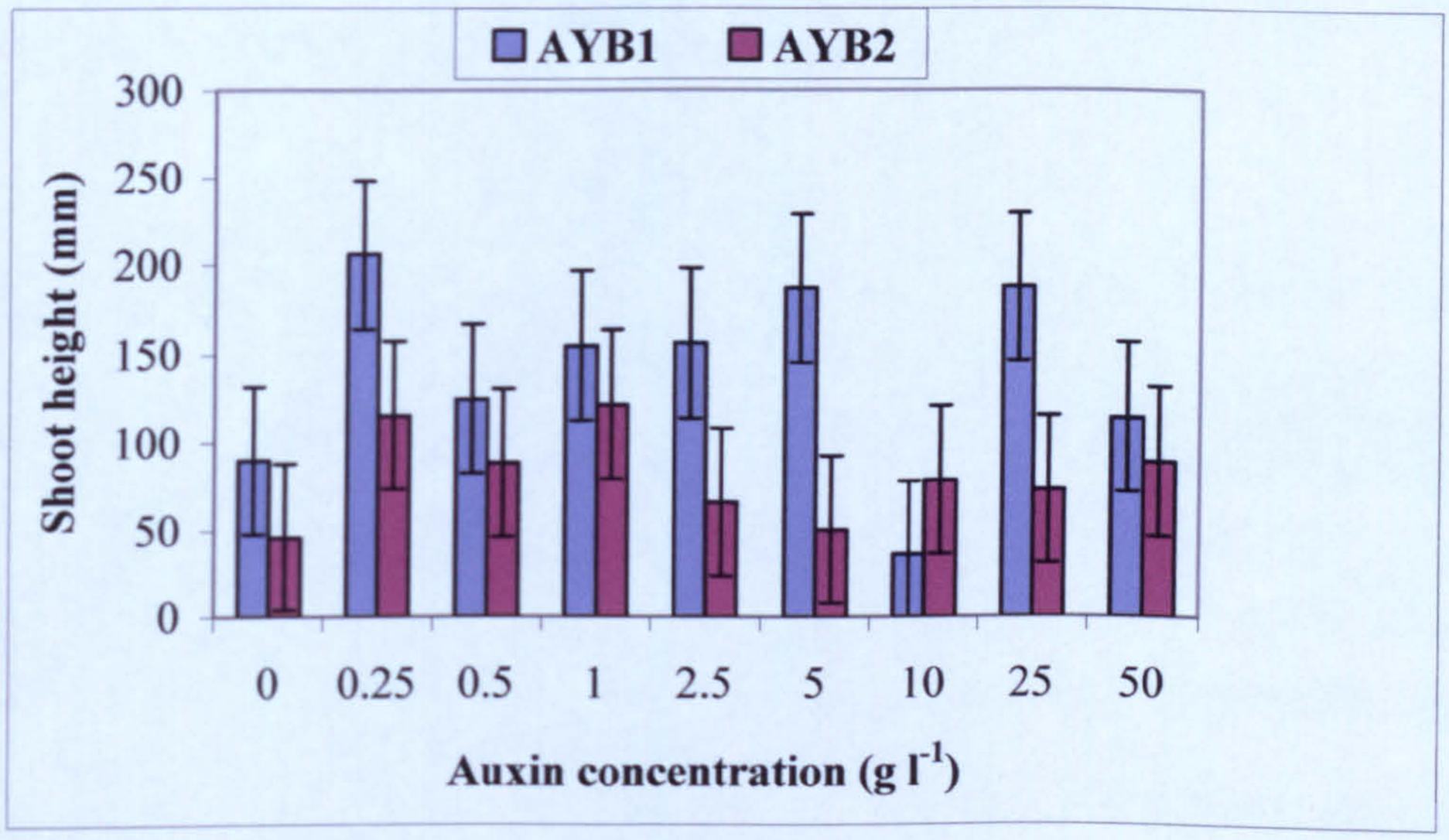

Figure 5.2. Effect of auxin concentration on bud growth (shoot height) of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=10) (Experiment 2)

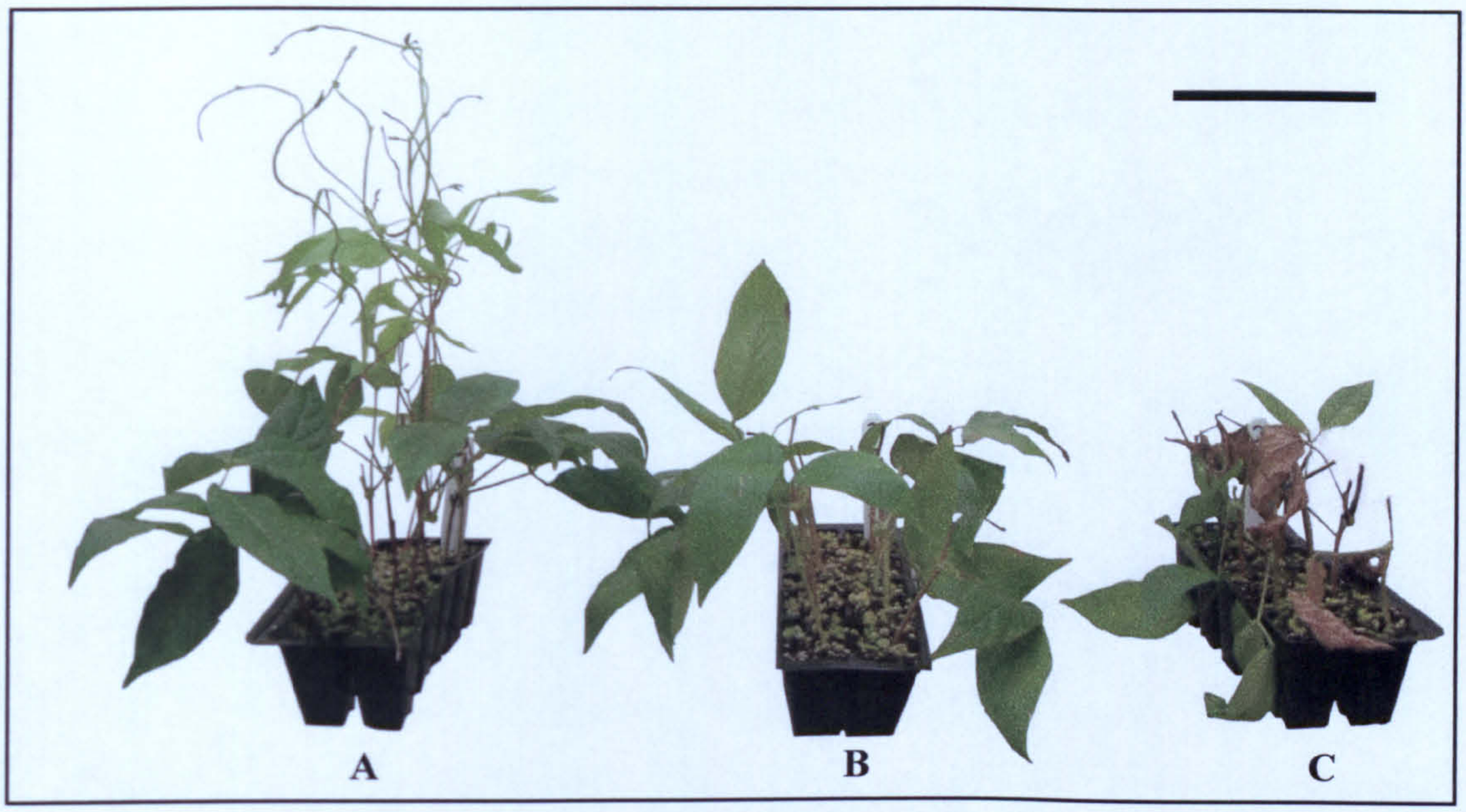

Plate 5.3 Effect of auxin concentration [A = 0 (control), B = 5.0 and C = 20 g l^{-1} IBA] on shoot height of AYB2 landrace. Bar = 10 cm.

5.2.2.5 Acclimation of rooted cuttings

Treatment of cuttings with auxin did not seem to have any effect on the ultimate survival in compost of plants derived from cuttings as most plants survived and grew into normal plants (Table 5.5). Plants derived from cuttings that were not treated with auxin and those receiving the highest auxin concentration (20.0 g l^{-1}) had 100% success in establishment for both landraces while 5.0 g l^{-1} auxin treatment had a slightly lower success proportion of 90.0 % and 79.0 % for AYB1 and AYB2 respectively.

Table 5.5 Establishment of AYB1 and AYB2 plants derived from cuttings treated with different concentrations of auxin (IBA).

Treatment Auxin (IBA g l^{-1})	Landrace					
	AYB1			AYB2		
	Potted plants	Surviving plants	% survival	Potted plants	Surviving plants	% survival
0	21	21	100.0	23	23	100.0
5.0	20	18	90.0	19	15	79.0
20.0	4	4	100.0	5	5	100.0

5.2.3 Discussion

From this study it is clear that AYB is amenable to propagation by stem cuttings and does not require auxins for root induction, which suggests that the cuttings contain enough endogenous auxins. However, auxin treatment was able to speed up rooting or give a more uniform root formation pattern as well as boost/increase the number of roots formed per cutting such that the combined effect of genotype and plant growth regulator were seen to play an influential role in adventitious root formation of AYB. Although the duration of the root initiation stage (>3 d) was the same for treated and untreated cuttings, application of auxin was found to induce a more synchronised rooting pattern in AYB such that more cuttings rooted earlier in the auxin treatments than in the control. *De novo* roots arose from the bark along the stem on cuttings treated with auxin while untreated cuttings grew roots from the wound/callus surfaces. Cuttings treated with auxin were also found to have more roots per cutting compared to control cuttings indicating that more potential root forming sites were triggered by auxin activity. This suggests that AYB has preformed or latent roots which require a stimulus, such as auxin, to start growth. However, this positive action of auxin was not without its limitations as cuttings that were treated with auxin experienced reduced root and shoot growth and, in the case of high concentrations, a high proportion of mortalities.

Several other studies have shown that exogenous application of auxin hastened the rate of rooting and increased root number of most species and, in some cases, relatively high concentrations resulted in inhibited root formation and growth, reduced bud/shoot growth and even mortalities (Badji *et al.*, 1991; Demeke *et al.*, 1992; Edson *et al.*, 1994; Ofori *et al.*, 1996; Shiembo *et al.*, 1996; Mesen *et al.*, 1997; De Andres *et al.*, 1999; Copes and Mandel, 2000; Fett-Neto *et al.*, 2001; Aminah, 2003; Ercisli *et al.*, 2003). Contrary to most findings, including those cited above, auxin application conferred no benefit in final rooting percentage as without auxin, a high proportion of cuttings rooted. Hartmann *et al.* (1997) indicated that reduced shoot growth with auxin could be due to alteration in sink and source dynamics where auxin modified assimilates partitioning in favour of the roots (sink). Since AYB rooted successfully without exogenous auxin, this suggests that it possesses adequate endogenous auxin such that reduction of bud

development/growth was due to elevation of total auxin to levels detrimental to bud growth.

The high root number induced by auxin did not confer any benefit on the establishment of rooted cuttings as both the treated and untreated cuttings successfully grew into normal plants. Survival of rooted stem cuttings is therefore, not controlled or affected by auxin. It was not possible to establish the benefit of shoot size achieved during rooting on the subsequent growth of the plants, although it is likely that growth of plants from auxin treated cuttings might be initially affected.

The following conclusions were made about the response of AYB cuttings to auxin treatment:

- AYB does not require exogenous auxin to form roots
- Auxin in low concentrations stimulates root formation and more roots per cutting
- High auxin concentration adversely affects root growth and bud development.

5.3 Effect of physiological status (leaf area) on rooting of AYB stem cuttings

Several factors including the carbohydrate status of cuttings have been shown to play an important role in the rooting of stem cuttings. Carbohydrates are necessary for plant tissues to produce adventitious roots and in tissue culture they are usually provided as sugar in the medium and in green tissues through photosynthesis (George and Sherrington, 1984). The physiological status of propagules such as the age and leaf area/size, plays an important role in the control of rooting. Nketiah *et al.* (1998) recorded higher rooting percentage of *Triplochiton scleroxylon* K. Schum from cuttings with large leaf area (100 cm^2) than smaller cuttings (30, 50 and 80 cm^2) using closed propagation boxes without mist. A large leaf area can also have a negative effect on root formation due mostly to water loss. Aminah *et al.* (1997) observed the highest rooting with small leaf area (15 cm^2) and decreased rooting of stem cuttings with larger leaf area (30 and 60 cm^2) in *Shorea leprosula* in enclosed polythene propagators (1×1×0.8 m) with a misting unit. The contradiction in the above findings is attributed to water loss through transpiration in larger propagators

while the smaller propagators tend to maintain more uniform high humidity around the cuttings.

Although it is common to reduce the size of large leaves so that cuttings occupy less space in propagation facilities, major reduction of leaf area and the mutual shading of leaves of crowded cuttings can reduce rooting and encourage diseases such as *Botrytis* (Hartmann *et al.*, 1997). Cuttings of AYB vary in size depending on their position on the vine with the smallest located towards the apex. In the trials reported early in this chapter, these small sized cuttings were discarded in case they did not root efficiently because of reduced photosynthetic capacity/carbohydrate reserves. It is therefore, important to evaluate effect of different propagule sizes on the rooting of AYB cuttings to avoid unnecessary wastage of material. This experiment tested the hypothesis that cuttings with large leaf size/area, root better than cuttings with smaller leaf size/area and that trimming of leaves interferes with rooting due to the associated physiological stress.

5.3.1 Materials and methods

Leaf cuttings were grouped into the following 3 classes (Plate 5.5) depending on leaf size/length of middle leaflet:

- small; 16 cm^2/≤ 6cm
- medium; 29 cm^2/ ≥6cm-≤ 8.5cm
- large; 65 cm^2/≥8.5 cm)

An additional treatment consisted of large leaf size class reduced by 50% by trimming the leaflets. Position of the cutting node on the vine was also noted to try to detect any positional effects. Aqueous IBA potassium salt at a concentration of 1.0 g l^{-1} (0.1%) (determined from results of Section 5.2) was applied to cuttings using the procedure described in Section 5.2.1 before placing them in the rooting medium. Three propagators were used, each containing 10 cuttings per treatment per landrace. In order to avoid interference with rooting, one box was set aside to monitor the rate of root formation by assessing the number of rooted cuttings per treatment at weekly intervals. In the third and last week of the experiment, the other two boxes were used to determine the number of rooted cuttings, number of roots per cutting and root length from 10 rooted cuttings selected randomly (with five cuttings selected from each box, where possible).

Plate 5.4 Three leaf sizes used to determine the effect of cutting size in rooting. Bar = 40 mm.

5.3.2 Results

5.3.2.1 Root formation

The position of a cutting on the parent vine did not seem to affect root formation in anyway since small sized cuttings located mostly on the upper position or closer to the apex and large sized cuttings on lower position performed similarly. Both of the AYB landraces were found to root readily with almost all the surviving cuttings rooted by the second week (Table 5.6). Root formation was not affected by leaf size.

Table 5.6 Effect of leaf size on rooting of single node stem cuttings of AYB1 and AYB2 landraces.

Treatment (leaf size)	Cumulative number of rooted cuttings (n=10)		Number of dead cuttings
	Week 1	Week 2	
AYB1			
Small	6	9	1
Medium	4	9	1
Trimmed	4	8	2
Large	9	9	1
AYB2			
Small	8	10	0
Medium	9	10	0
Trimmed	7	10	0
Large	7	10	0

5.3.2.2 Root number

There was a significant effect of leaf size ($F_{(3,70)} = 3.23$, $p<0.05$) on the number of roots per rooted cutting (Table 5.7). In AYB2, trimmed leaf cuttings produced the most roots. Root number formed per cutting did not differ significantly between the landraces.

5.3.2.3 Root length

Differences in root length between treatments and between landraces were not significant (Table 5.7).

Table 5.7 Effect of leaf size on mean number of roots per cutting and mean root length of AYB1 and AYB2 landraces. (n=10)

Factor	Number of roots per cutting		Root length (longest root in mm)	
	AYB1	AYB2	AYB1	AYB2
Leaf size				
Small	20.4	18.2	116.3	139.1
Medium	17.7	14.9	145.7	161.2
Trimmed	23.5	26.7	96.5	129.9
Large	21.1	16.0	135.5	132.4
s.e.d.	4.18		25.69	
d.f.	70		70	
Significance (5%)				
Landrace	0.415		0.186	
Leaf size	0.027		0.178	
Interaction	0.552		0.783	

5.3.3 Discussion

Leaf size did not seem to have any influence on the rooting of AYB. In contrast with observations made for other species (Aminah *et al.*, 1997; Nketiah *et al.*, 1998), no clear differences were detected in the rooting of the AYB landraces and cuttings with different leaf sizes were suitable as propagules. This is not unexpected because AYB is herbaceous and many relatively succulent non-woody plants that are rooted easily by herbaceous cuttings follow a similar response (Hartmann *et al.*, 1997). Although smaller cuttings were mostly derived from the apical positions of the vine, this did not seem to have any bearing on the rate of root formation as rooting was uniform amongst the different cuttings from different positions on the vine.

Reducing the leaf size by trimming seemed to enhance mortality of the cuttings. Although a common practice, reduction of the size of leaves in order to occupy less space in propagating facilities encourages diseases such as *Botrytis* (Hartmann *et al.*, 1997). Trimming leaves did not seem to affect rooting of AYB as all the surviving trimmed cuttings were also able to root, just like the cuttings with intact leaves. There was no trend on root number and root length associated with leaf size of cuttings, although cuttings that yielded the highest root number appeared to have relatively shorter roots which might imply some kind of competition due to increased sink size thus impacting on individual root growth. This indifference of root number and root length towards leaf size is in contradiction to what has been reported for other plant species. For example, Ofori *et al.* (1996) reported an increase in root number with increase in leaf area of cuttings of *Milicia excelsa*.

It can be concluded that leaf size does not have a bearing on rooting of AYB and that all of the types of cuttings used in this trial can be successfully used as propagules for propagation of AYB. It was also found that trimming of leaves does not negatively affect rooting although there were mortalities associated with it due possibly to fungal diseases. Further trials involving trimming of leaves should include some treatments with fungicides to counteract fungal infection.

CHAPTER 6

IN VITRO PROPAGATION

6.1 Introduction

In vitro propagation can be divided into four stages, (i) the establishment of axenic cultures, (ii) the proliferation or multiplication of shoots, (iii) the production of plantlets with roots and (iv) re-establishment of plants in compost (Debergh and Read, 1991). The listing of the four stages above is not prejudicial to stage 0, i.e. the preparative stage in which mother plants are raised under hygienic conditions in order to limit contamination problems.

Shoot regeneration from nodal explants can be used to achieve mass propagation of clonal progeny by either allowing growth of multiple shoots (axillary shoot growth), or a single elongated shoot bearing discrete and separated nodes that can be cut into nodal pieces and further subcultured to obtain more shoots. Good growth of plant tissues *in vitro* is obtained using protocols that provide optimal conditions for physiological functions of the explant. Naturally occurring and synthetic cytokinins are used to stimulate growth and development in tissue cultures (Pierik, 1987), and they are recognised to play an opposing role to auxins, that of inhibiting apical dominance as well as retarding senescence (Moore, 1989). Such stimulation of *in vitro* growth has been recorded by many researchers with recognisable differences between the various cytokinins used. Other plant growth regulators, such as gibberellins and auxin transport inhibitors, have been used to stimulate regeneration in *in vitro* cultures (Pattnaik and Chand, 1997; Chitra and Padmaja, 1999; Nakano *et al.*, 2000).

Rooting of *in vitro* derived plants is an important step in micropropagation as the ultimate goal is to grow the plants *ex vitro*. Although some species easily form adventitious roots on shoots produced *in vitro*, for others it may be necessary to apply certain treatments before they can grow roots (George and Sherrington, 1984). Auxins have been recognised to play an important role in the promotion of root growth in many plant species (Nikam and Shitole, 1997). Cytokinins used to induce

shoot growth frequently inhibit root formation and usually such shoots do not form roots *in vitro* until they are cultured on a medium with or without auxins. The potency of auxin in formation/induction of roots differs with auxin type, auxin concentration as well as plant genotype. Gibberellins are also naturally occurring growth substances that induce growth, but are not generally known to favour organ initiation (Rout *et al.*, 2000) and usually inhibit adventitious root formation (Pierik, 1987). Rooting of species previously known to be slow to produce roots has been achieved on explants that were exposed to gibberellin biosynthesis inhibitors (George and Sherrington, 1984; Mckinless and Alderson, 1993). Such chemicals have the capability to block endogenous gibberellin biosynthesis in cultures and thus promote root growth.

Studies with other grain legumes such as cowpea (*Vigna unguiculata* L.) (Pellegrineschi, 1997; Brar *et al.*, 1997; Brar *et al.*, 1999), common bean (*Phaseolus vulgaris* L.) (Nagy *et al.*, 1991; Santalla *et al.*, 1998; Zambre *et al.*, 1998) and black gram (*Vigna mungo* L.)(Geetha *et al.*, 1997a,b) have demonstrated the effectiveness of *in vitro* techniques as propagation tools, thus offering hope for application in the propagation of AYB.

6.2 Establishment of axenic shoot cultures (culture initiation)

For the rapid multiplication of shoots *in vitro*, it is important to establish axenic cultures from which explants can be subcultured in the shoot multiplication stage and these shoots or axillary buds can then be rooted or induced to elongate respectively.

6.2.1 Effect of BAP on shoot induction and growth

Various natural and synthetic cytokinins are used to stimulate growth and development in tissue culture (Pierik, 1987). Amongst the cytokinins used in tissue culture, BAP produced the highest efficacy in shoot/bud proliferation (Yang and Read, 1996; Brar *et al.* 1997). Increased concentration of BAP from 1.0 to 5.0 mg l^{-1} resulted in increased shoot bud production of black gram (*Vigna mungo*) (Geetha *et al.*, 1997b). In the same study, a high frequency of shoot-bud differentiation was observed at 3.0 mg l^{-1}. In some instances, high concentrations were observed to favour callus proliferation and inhibited shoot growth. High concentrations of BAP caused browning of cultures, less vigorous thin shoots and shoot tip decay of

chickpea (*Cicer arietinum*) (Polisetty *et al.*, 1997).

Two preliminary experiments were carried out to establish the suitable BAP concentration for the initiation of shoot growth from stem nodal segments of AYB1, AYB2, AYB3 and AYB4. The experiment tested the hypothesis that cytokinin is required for *in vitro* shoot growth and that the degree of stimulation vary with concentration. Due to failure to germinate, AYB5 landrace was not included in this experiment.

6.2.1.1 Materials and methods

Stem nodal explants were surface sterilised as described in Chapter 3, Section 3.4.4 and cultured separately. The following media treatments within the range of common or average concentrations used in other studies were used with five replicate explants (cultures) per treatment:

Experiment 1: BAP at 0.25, 0.5, 1.0, 1.5 and 2.5 mg l^{-1}.

Experiment 2: Half strength MS lacking BAP and full strength MS + 0, 0.25, 0.5, 1.0, 1.5 and 2.5 mg l^{-1} BAP.

The cultures were incubated for 5 weeks in conditions described in Chapter 3, Section 3.7.1, after which shoot growth and callus production were recorded.

6.2.1.2 Results

In both experiments, more than 90% of the cultures of all the genotypes were free of contamination following the surface sterilisation of explants in 10 % (v/v) "Domestos" for 20 minutes (Table 6.1). Overall, there was a positive effect of BAP on the induction and growth of shoots of the AYB landraces. However, the stimulation of *in vitro* growth differed in terms of shoot number, shoot length and callus proliferation. The shoots were observed to be arising from axillary buds and there was no evidence of adventitious shoots formation either from stem or the accompanying callus.

Table 6.1 Response of AYB1, AYB2, AYB3 and AYB4 landraces to surface sterilization with 10 % (v/v) "Domestos" for 20 minutes.

Landrace	Experiment 1 (n=25)		Experiment 2 (n=35)	
	Number of aseptic cultures	% of aseptic explants	Number of aseptic cultures	% of aseptic explants
AYB1	23	92	33	94.29
AYB2	25	100	34	97.14
AYB3	25	100	35	100.00
AYB4	23	92	34	97.14

Significantly more shoots were produced in medium enriched with high BAP concentrations in both experiments [($F_{(4,80)}$ = 2.80, p<0.05) and ($F_{(6,108)}$ = 31.96, p<0.001) for Experiments 1 and 2 respectively] (Tables 6.2 and 6.3). However, in both experiments the trend was similar with an increase in shoot number concomitant with BAP concentration. Media without BAP did not stimulate any shoot formation. Shoot numbers varied significantly ($F_{(3,80)}$ = 11.84, p<0.001) and ($F_{(3,108)}$ = 6.30, p<0.001) between the different landraces in Experiments 1 and 2 respectively suggesting a strong genotypic control (Tables 6.2 and 6.3). In Experiment 1, AYB1 and AYB2 produced more shoots than AYB3 and AYB4 whereas there was a marked improvement in performance of AYB4 in Experiment 2.

In Experiment 2, addition of 0.25 mg l^{-1} BAP to MS medium had a positive effect on shoot height ($F_{(6,108)}$ = 41.50, p<0.001) but increasing the level further reduced this effect (Figure 6.1 and Plate 6.1). Thus, BAP at 0.25 and 0.5 mg l^{-1} was determined to be suitable for growth of AYB explants *in vitro*. Explants cultured in half strength and full strength MS media without BAP made little or no growth.

Table 6.2 Effect of BAP concentration on mean number of shoots of AYB1, AYB2, AYB3 and AYB4 landraces regenerated *in vitro*. (n=5) (Experiment 1)

Treatment	**Shoot number**			
	AYB1	AYB2	AYB3	AYB4
BAP concentration (mg l^{-1})				
0.25	1.8	2.2	1.2	1.6
0.50	2.8	2.2	1.6	1.2
1.00	2.0	2.2	1.2	1.2
1.50	2.0	2.6	1.8	2.0
2.50	2.4	2.2	1.8	2.0
s.e.d.	0.36			
d.f.	80			
Significance (0.05)				
Landrace	<0.001			
BAP concentration	0.031			
Landrace × BAP concentration	0.231			

Table 6.3 Effect of BAP concentration on mean number of shoots of AYB1, AYB2, AYB3 and AYB4 landraces regenerated *in vitro*. (n=5) (Experiment 2)

Treatment	**Shoot number**			
	AYB1	AYB2	AYB3	AYB4
BAP concentration (mg l^{-1})				
0 (half MS)	0.8	1.6	0.4	0.5
0 (full MS)	1.0	1.4	0.0	1.2
0.25	2.2	2.0	1.6	1.6
0.50	2.0	3.0	2.4	2.2
1.00	3.3	3.0	2.2	4.0
1.50	3.2	4.2	2.8	3.6
2.50	3.0	4.4	3.6	4.8
s.e.d.	0.61			
d.f.	108			
Significance (0.05)				
Landrace	<0.001			
BAP concentration	<0.001			
Landrace × BAP concentration	0.294			

Plate 6.1 Effect of BAP concentrations [A; 0.25, B; 0.5, C; 1.0, D; 1.5, E; 2.5, F; 0 (half MS) and G; 0 mg l^{-1} (Full MS)] on shoot proliferation and growth of AYB landraces. Bar = 25 mm.

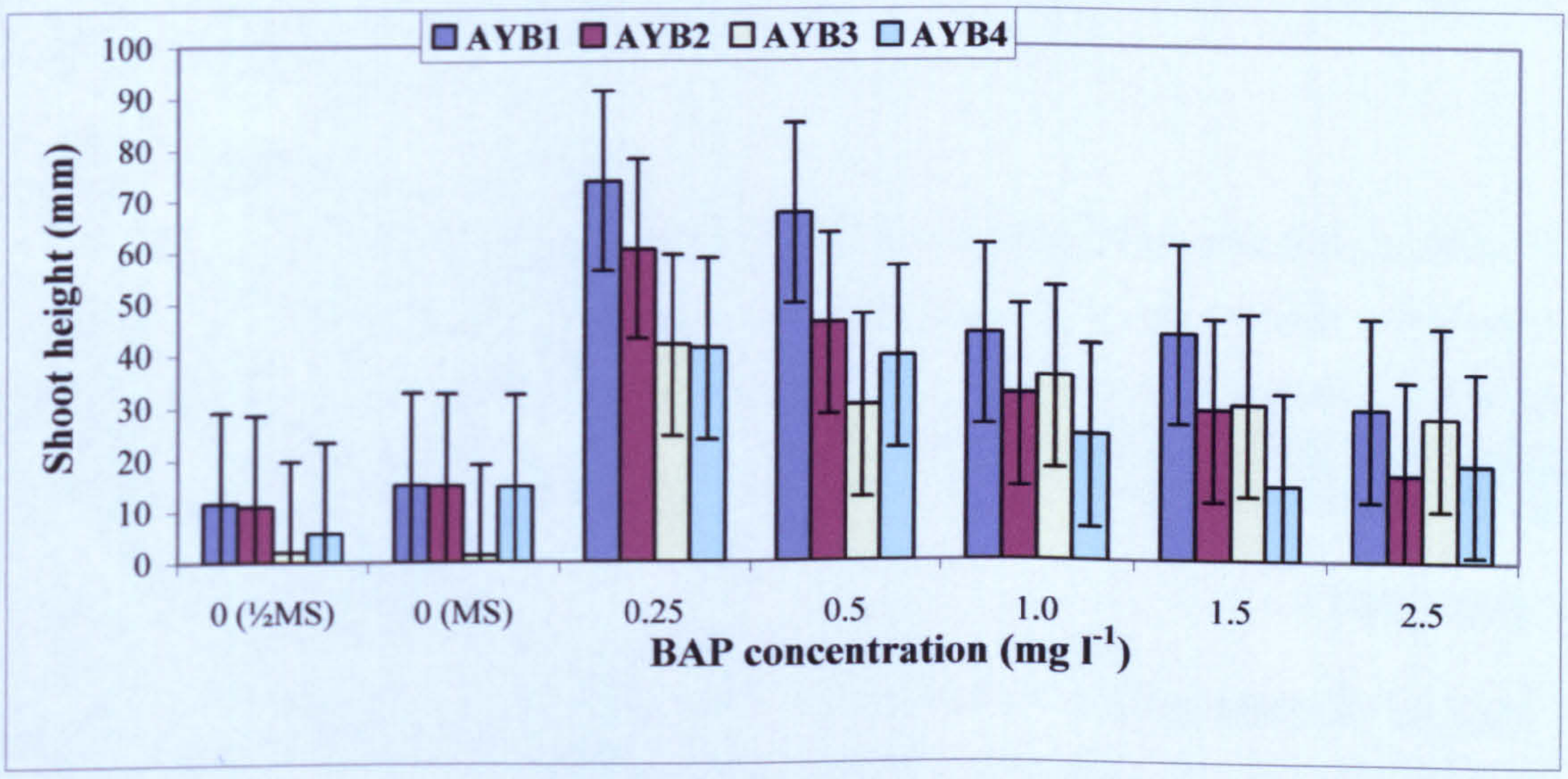

Figure 6.1 Effect of BAP concentration in MS medium on mean shoot height of AYB1, AYB2, AYB3 and AYB4 landraces. Error bars = 2 *s.e.d.* (n=5) (Experiment 2)

Large amounts of callus ($F_{(6,108)}$ = 57.14, p<0.001) were produced by explants cultured on media containing different concentrations of BAP but less was produced by explants cultured on media without cytokinin (Figure 6.2 and Plate 6.1). The interaction between landrace and BAP concentration was significant ($F_{(3,108)}$ = 2.21, p<0.01). AYB1 produced the largest amount of callus at all BAP concentrations and there was a trend towards more callus with low BAP concentration.

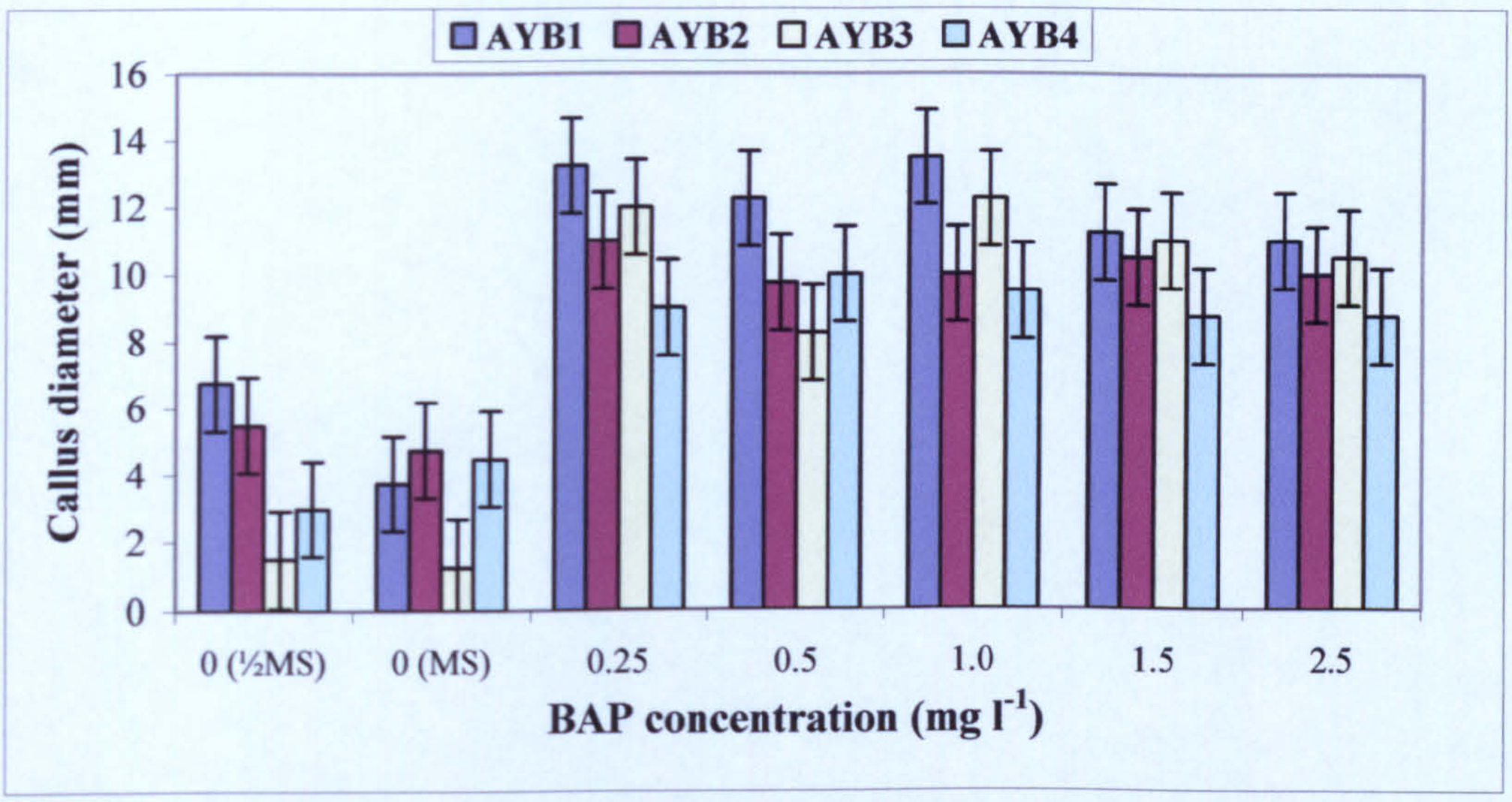

Figure 6.2 Effect of BAP concentration in MS medium on mean callus size (diameter) produced by AYB1, AYB2, AYB3 and AYB4 landraces. Error bars = 2 *s.e.d.* (n=5) (Experiment 2)

6.2.1.3 Discussion

Although AYB does not have an erect stem, its climbing habit makes it possible to keep it away from the ground, thus eliminating contact with soil-borne microorganisms. Therefore, in the preparative stage for micropropagation, growing the mother/stock plants on a trellis/support enhances success with establishment of axenic cultures from explants.

Cytokinin was found to be essential for the growth of AYB *in vitro* but, at high concentrations, elongation of shoots was adversely affected. The increase in shoot number and reduced shoot elongation associated with higher BAP concentration is in agreement with observations made by Polisetty *et al*. (1997) in chickpea and Girija *et al*. (1999) in firecracker plant/flower (*Crossandra infundibuliformis*). The different effects of BAP concentration on shoot proliferation and elongation suggest that a two stage approach for the *in vitro* regeneration of AYB should be adopted, where separate media high and low in cytokinin are used for shoot proliferation and elongation respectively. The absence of a BAP concentration effect on callus production contradicts several findings with other crops. Avenido and Hattori (2000) recorded a significant increase in callus from hypocotyls of adzuki bean (*Vigna angularis*) when the BA concentration was increased twofold. The callus produced in all cultures grown on media with BAP may interfere with shoot regeneration

through competition for growth requirements, and thus needs to be reduced if direct shoot proliferation from meristematic tissue is to be achieved. The differences observed between the landraces suggest genotypic control in *in vitro* regeneration as observed in other species (Polisetty *et al.*, 1997; Avenido and Hattori, 2000).

6.2.2 Effect different cytokinins on shoot induction and growth

The effect of cytokinins is dependent on many factors, including concentration and type of cytokinin (Pierik, 1987). An experiment was carried out to identify the most suitable cytokinin for *in vitro* regeneration of AYB landraces. Due to their overall positive response in the initial experiments (Section 6.2.1), AYB1 and AYB2 were selected for this trial and other subsequent ones. Amongst the cytokinins used in tissue culture, BAP produced the highest efficacy in shoot/bud proliferation (Yang and Read, 1996; Brar *et al.*, 1997). The following experiment was carried out to test the hypothesis that cytokinins differ in their activity to stimulate *in vitro* shoot regeneration.

6.2.2.1 Materials and methods

Stem nodal explants of AYB1 and AYB2 landraces were surface sterilised as described in Chapter 3, Section 3.4.4 and cultured on MS medium containing TDZ, BAP or 2iP at 0.25, 0.5, 1.0 and 1.5 mg l^{-1}. There were 5 explants (cultures) per treatment and they were incubated as described in Chapter 3, Section 3.7.1 for 5 weeks after which shoot growth and callus production were recorded.

6.2.2.2 Results

There were significant differences in the effects of the cytokinins on shoot number, shoot height and callus proliferation (Table 6.4 and Figures 6.3 and 6.4). Differences between the landraces of AYB were not significant, however, there were some interactions between cytokinin and landrace for some parameters.

Visual observation found the shoots to be of axillary buds origin. Significantly ($F_{(2,92)} = 73.20, p<0.001$) more shoots were produced on medium supplemented with TDZ than with 2iP or BAP (Table 6.4). Shoot number was greater at low TDZ concentrations, whereas it increased with an increase in concentration of BAP and 2iP. Differences in the numbers of shoots stimulated by 2iP and BAP at the lowest

concentration were not significant. Although there was a highly significant ($F_{(6,92)}$ = 5.19, p<0.001) interaction between cytokinin type and concentration, differences between concentrations were not significant (Table 6.4). The interaction between landrace, cytokinin type and concentration was significant ($F_{(6,92)}$ = 2.40, p<0.05) with AYB1 producing more shoots at 0.25 mg l^{-1} TDZ whereas AYB2 had more shoots at 0.5 mg l^{-1}.

Both type and concentration of cytokinin in the media significantly [($F_{(2,92)}$ = 184.60, p<0.001) and ($F_{(3,92)}$ = 3.32, p<0.05) respectively] influenced shoot height (Figure 6.3). Compared with TDZ and 2iP, BAP produced shoots that were significantly taller, however, there was a significant interaction between cytokinin, concentration of cytokinin and genotype. Shoot height varied significantly ($F_{(6,92)}$ = 2.28, p<0.05) between landraces and from cytokinin to cytokinin at different concentrations (Figure 6.3).

Table 6.4 Effect of type of cytokinin and cytokinin concentration on mean number of shoots of AYB1 and AYB2 landraces regenerated *in vitro*. (n=5)

Factor	AYB1			AYB2		
	BAP	2iP	TDZ	BAP	2iP	TDZ
Cytokinin concentration (mg l^{-1})						
0.25	1.0	1.0	3.2	1.0	1.0	2.6
0.50	1.4	1.2	2.0	1.5	1.0	3.2
1.00	1.6	1.0	2.0	1.8	1.2	2.0
1.50	1.6	1.6	2.3	1.8	1.0	2.2
s.e.d.	0.32					
d.f.	6					
Significant (0.05)						
Landrace	0.751					
Cytokinin	<0.001					
Cytokinin concentration	0.656					
Landrace × cytokinin	0.384					
Landrace × cytokinin conc.	0.105					
Cytokinin × cytokinin conc.	<0.001					
Landrace × cytokinin × cytokinin conc.	0.034					

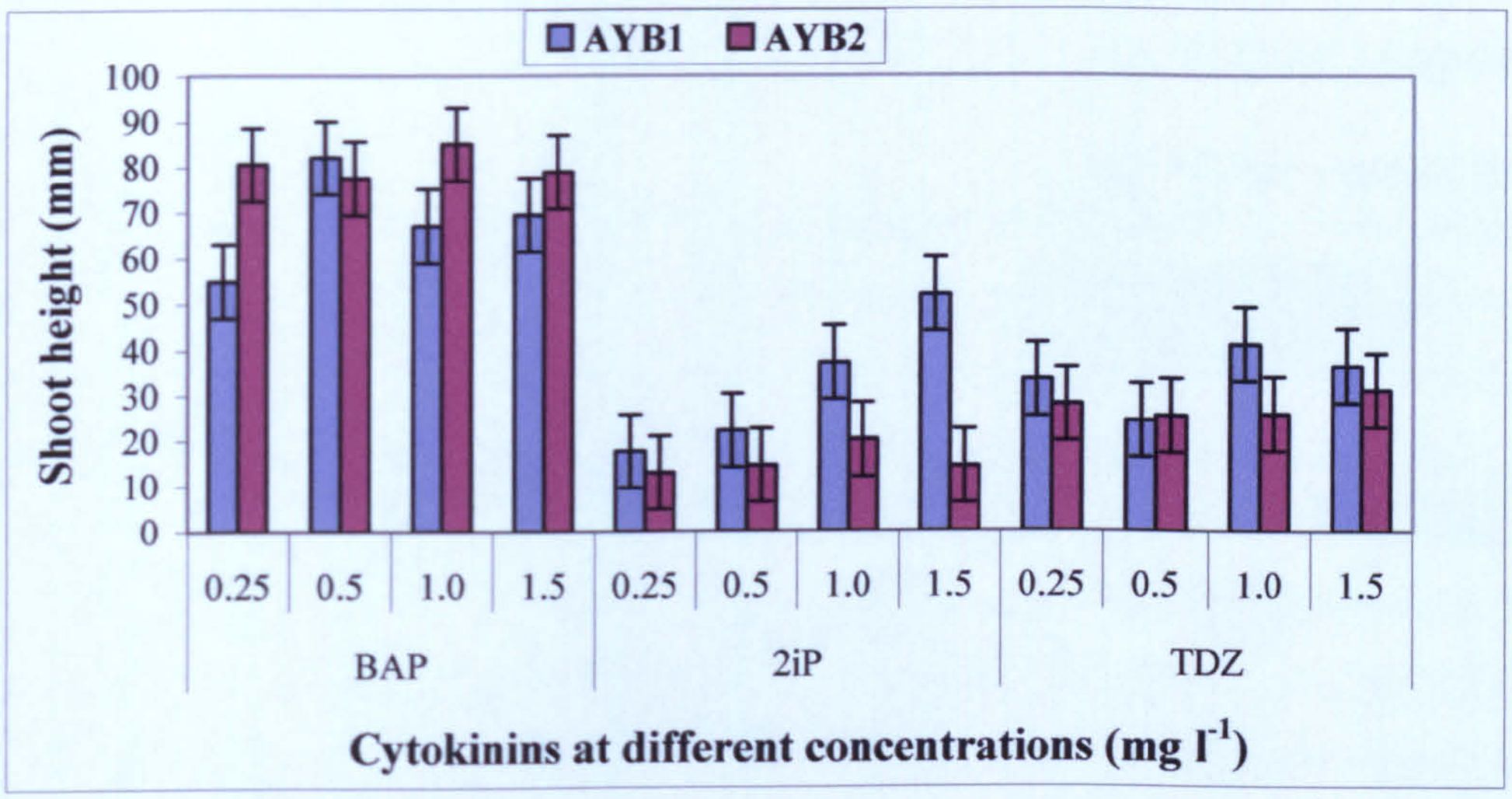

Figure 6.3 Effect of cytokinin type and concentration on mean shoot height of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5)

Shoot regeneration from AYB was accompanied by excessive callus growth. All of the cytokinins tested stimulated callus, but the amounts differed significantly ($F_{(2,92)}$ = 74.03, p<0.001) (Figure 6.4). TDZ produced the largest amount of callus while 2iP produced the least. Callus proliferation was not dependent on genotype. However, the response of individual landraces varied between cytokinins and cytokinin concentrations as shown by the significant ($F_{(6,92)}$ = 3.04, p<0.01) interaction between landrace, cytokinin and cytokinin concentration. Less callus was produced by all landraces in the presence of 2iP than with BAP or TDZ. AYB2 was the least prolific landrace on 2iP media and highest on TDZ media (Figure 6.4).

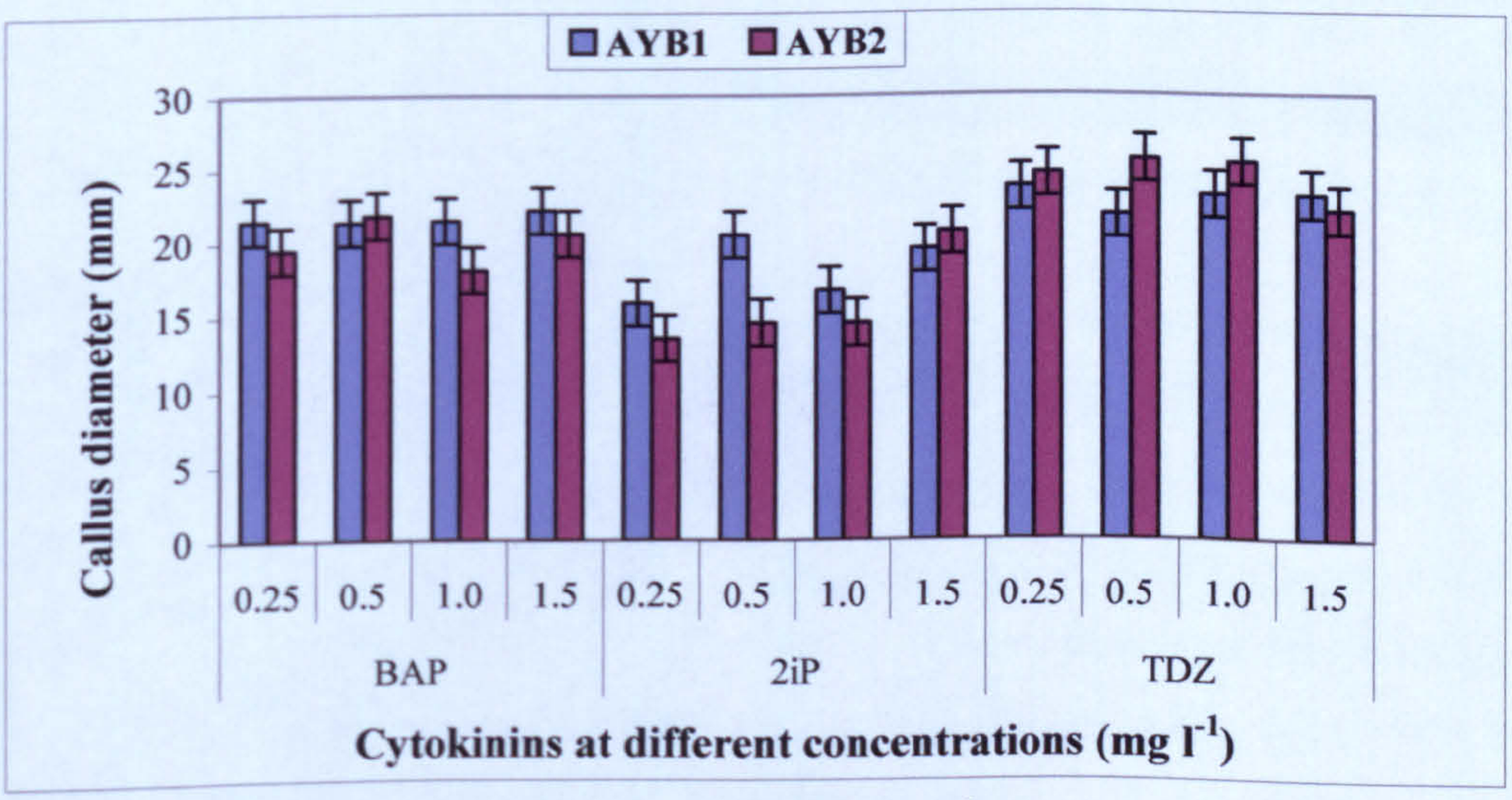

Figure 6.4 Effect of cytokinin type and concentration on mean callus size (diameter) produced by AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5)

6.2.2.3 Discussion

Cytokinins tested in this experiment were shown to differ significantly in their action on the type of growth invoked in AYB, affirming previous findings on the potency of different cytokinins. TDZ performed better in terms of number of shoots compared with BAP and 2iP, while shoot elongation was better in BAP enriched media. Naik *et al.* (1999) observed a similar action of TDZ on pomegranate (*Punica granatum*) and attributed this to its high activity that can be inhibitory to shoot development. BAP has been observed to be a better cytokinin for shoot development as well as shoot induction (Yang and Read, 1996; Brar *et al.*, 1997; Pattnaik and Chand, 1997; Tavares *et al.*, 1998; Chitra and Padmaja, 1999). The low potency of 2iP with respect to shoot induction and development was also observed for callus proliferation, which suggests that it could be selected where cultures low in callus are required, although it may need to be used at a slightly higher concentration.

6.2.3 Effect of media salts on shoot induction and growth

Sufficient nutrient salts are essential to avoid limited culture growth due to nutrient stress. Different formulations for media are available and those commonly used are MS medium (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and Woody Plant Medium (McCown and Lloyd, 1981). MS medium is the most popular as most plants react favourably to it, but its salt content is too high for some plants (Pierik, 1987).

Two experiments were carried out to test the hypothesis that the three commonly used media salts, Woody Plant (WPM), Gamborg B5 and Murashige and Skoog (MS), do not differ in efficiency to supported shoot regeneration of AYB *in vitro*.

6.2.3.1 Materials and methods

Surface sterilised stem nodal explants (Chapter 3, Section 3.4.4) were cultured on WPM, B5 and MS media containing BAP at 1.0 mg l^{-1}. Both experiments were run as a complete randomised design with seven and nine replicates (cultures) for each treatment in Experiments 1 and 2 respectively. The cultures were incubated for five weeks under conditions described in Chapter 3, Section 3.7.1, after which shoot growth and callus production were recorded.

6.2.3.2 Results

In both experiments, all the shoots were observed to arise from axillary buds and none from other parts of stem segment or the accompanying callus. In Experiment 1, the number of shoots produced differed significantly ($F_{(2,36)} = 4.04$, $p<0.05$) between media but not between landraces (Table 6.5). In overall, MS gave more shoots than B5 and WPM but differences were only significant between MS and WPM. In Experiment 2, the difference in the number of shoots due to medium was also significant ($F_{(2,36)} = 4.79$, $p<0.05$) with MS still producing more shoots and WPM producing the least (Table 6.6). The landraces also differed significantly ($F_{(1,48)} = 8.64$, $p<0.01$) with more shoots produced by AYB1 (Table 6.6). In Experiment 1, shoot fresh weight differed between landraces ($F_{(1,36)} = 29.44$, $p<0.001$) and between media ($F_{(2,36)} = 23.63$, $p<0.001$) with AYB2 shoots being the heaviest and those produced on WPM the lightest (Table 6.5). However, in Experiment 2, shoot weight was only affected ($F_{(2,48)} = 20.90$, $p<0.001$) by medium with MS producing the heaviest and WPM the lightest (Table 6.6).

Shoot height was affected by genotype ($F_{(1,36)} = 9.63$, $p<0.01$) in Experiment 1 but not by growth medium where AYB2 produced taller shoots than AYB1 (Figure 6.5). In Experiment 2 it was affected by medium ($F_{(2,48)} = 3.36$, $p<0.05$) with taller shoots produced on WPM than on MS and B5 (Figure 6.6 and Plate 6.2) Both landrace and medium had significant effects [($F_{(1,48)} = 59.27$, $p<0.001$) and ($F_{(2,48)} = 34.98$, $p<0.001$) respectively] on number of nodes (Table 6.6). Shoots of AYB1 possessed more nodes than those of AYB2, and shoots produced on MS had more nodes than those on B5 and WPM.

Table 6.5 Effect of different media salts on mean shoot number, mean shoot weight and mean callus weight of AYB1 and AYB2 landraces regenerated *in vitro*. (n=7) (Experiment 1)

Factor	**Shoot number**		**Shoot weight (g)**		**Callus weight (g)**	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Medium						
MS	1.9	2.4	0.148	0.204	3.52	2.57
B5	2.1	1.6	0.123	0.214	1.52	2.29
WPM	1.1	1.6	0.076	0.109	1.28	1.38
s.e.d.	0.40		0.0192		0.689	
d.f.	2		2		2	
Significance (0.05)						
Landrace	0.536		<0.001		0.949	
Medium	0.026		<0.001		0.004	
Landrace × medium	0.098		0.107		0.217	

Table 6.6 Effect of different media salts on mean shoot number, mean shoot weight, mean number of nodes and mean callus weight of AYB1 and AYB2 landraces regenerated *in vitro*. (n=9) (Experiment 2)

Factor	Shoot number		Shoot weight (g)		Number of nodes		Callus weight (g)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Medium								
MS	2.22	1.56	0.143	0.138	12.33	8.78	2.09	2.34
B5	1.90	1.67	0.126	0.103	10.78	7.56	1.58	1.70
WPM	1.56	1.22	0.083	0.074	7.89	4.00	1.00	0.97
s.e.d.	0.240		0.1354		0.800		0.225	
d.f.	2		2		2		2	
Significance (0.05)								
Landrace	0.005		0.125		<0.001		0.392	
Medium	0.013		<0.001		<0.001		<0.001	
Landrace × medium	0.402		0.657		0.841		0.687	

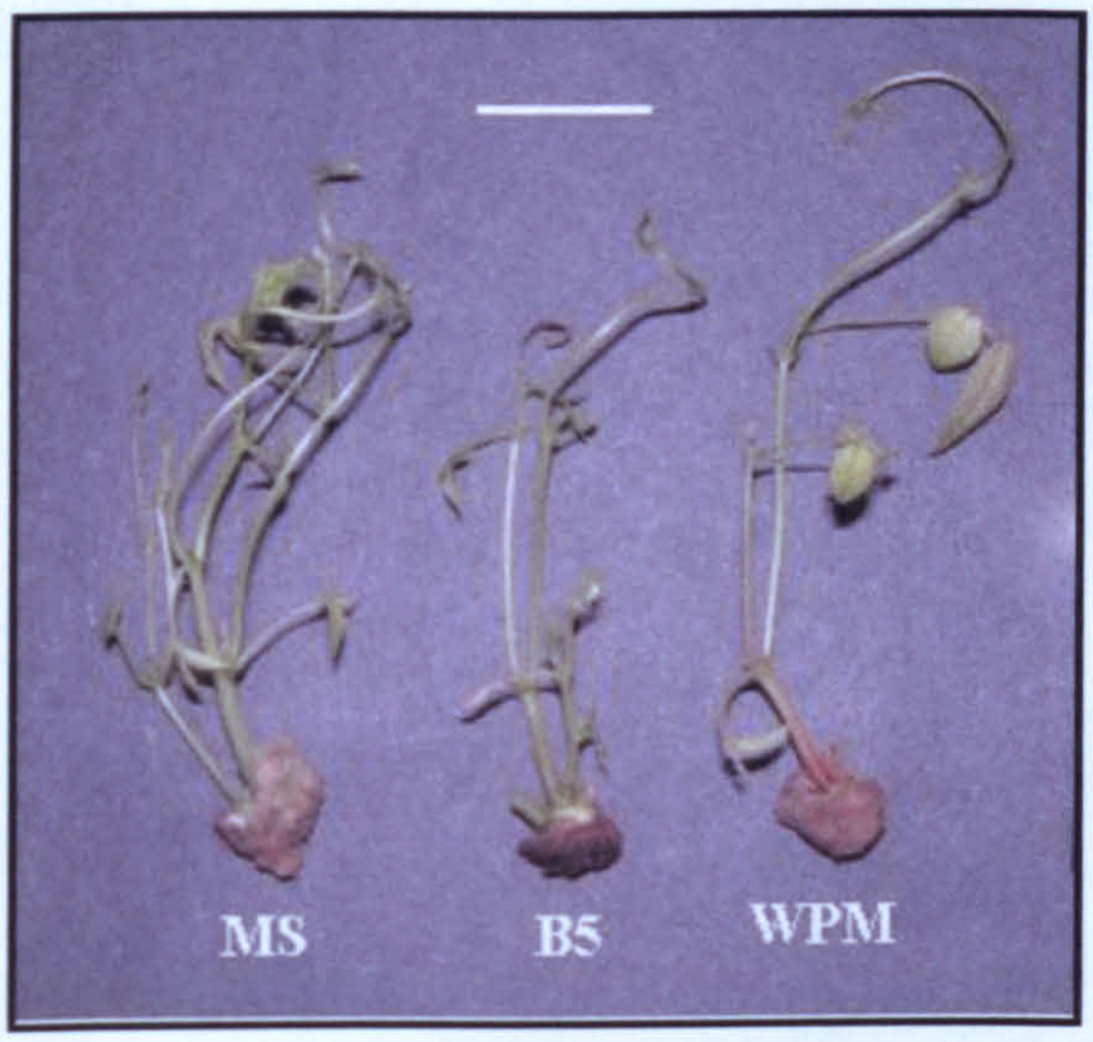

Plate 6.2 Effect of different media salts on *in vitro* shoot regeneration of AYB1 and AYB2. Bar = 20 mm.

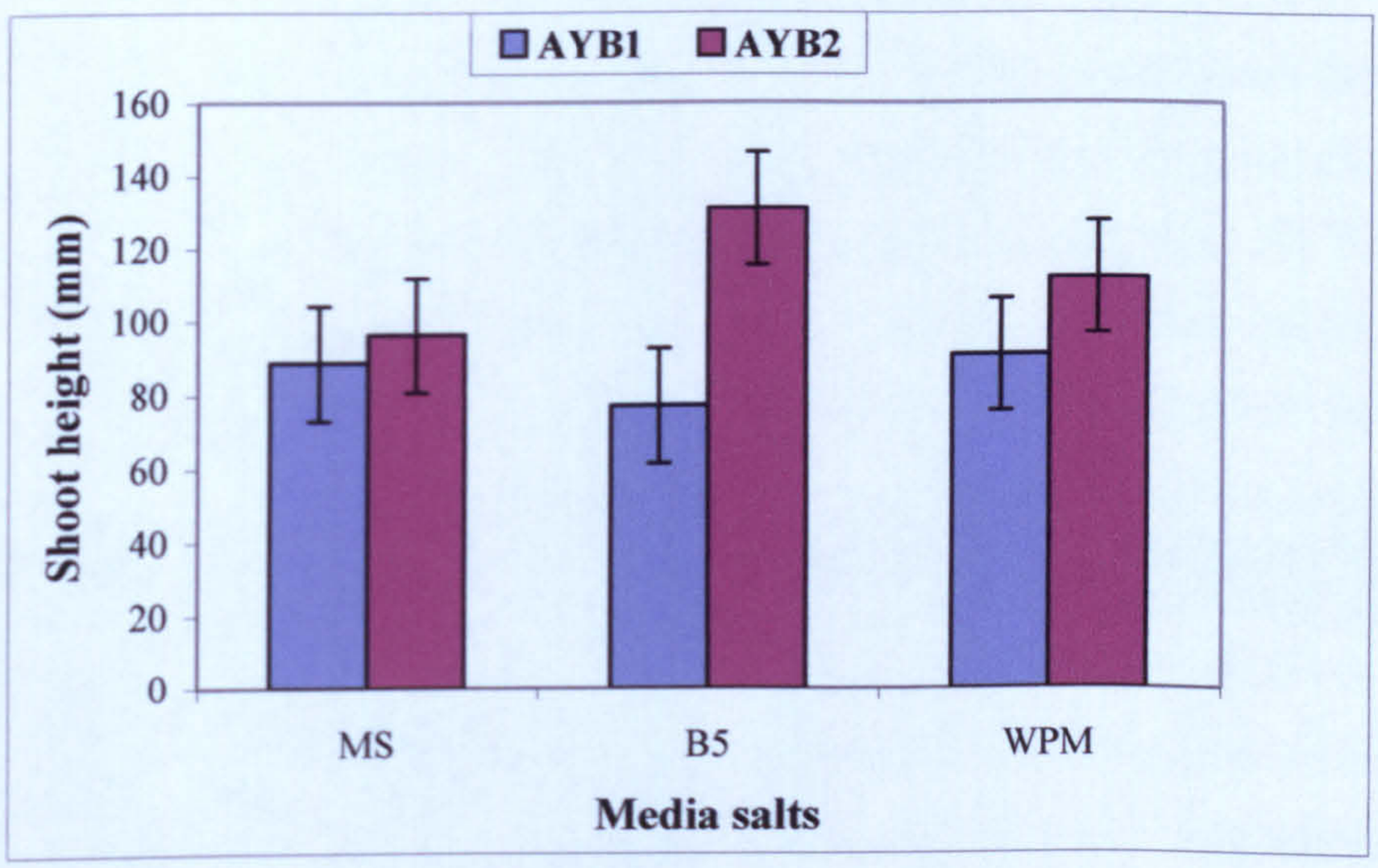

Figure 6.5 Effect of different media salts on mean shoot height of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=7) (Experiment 1)

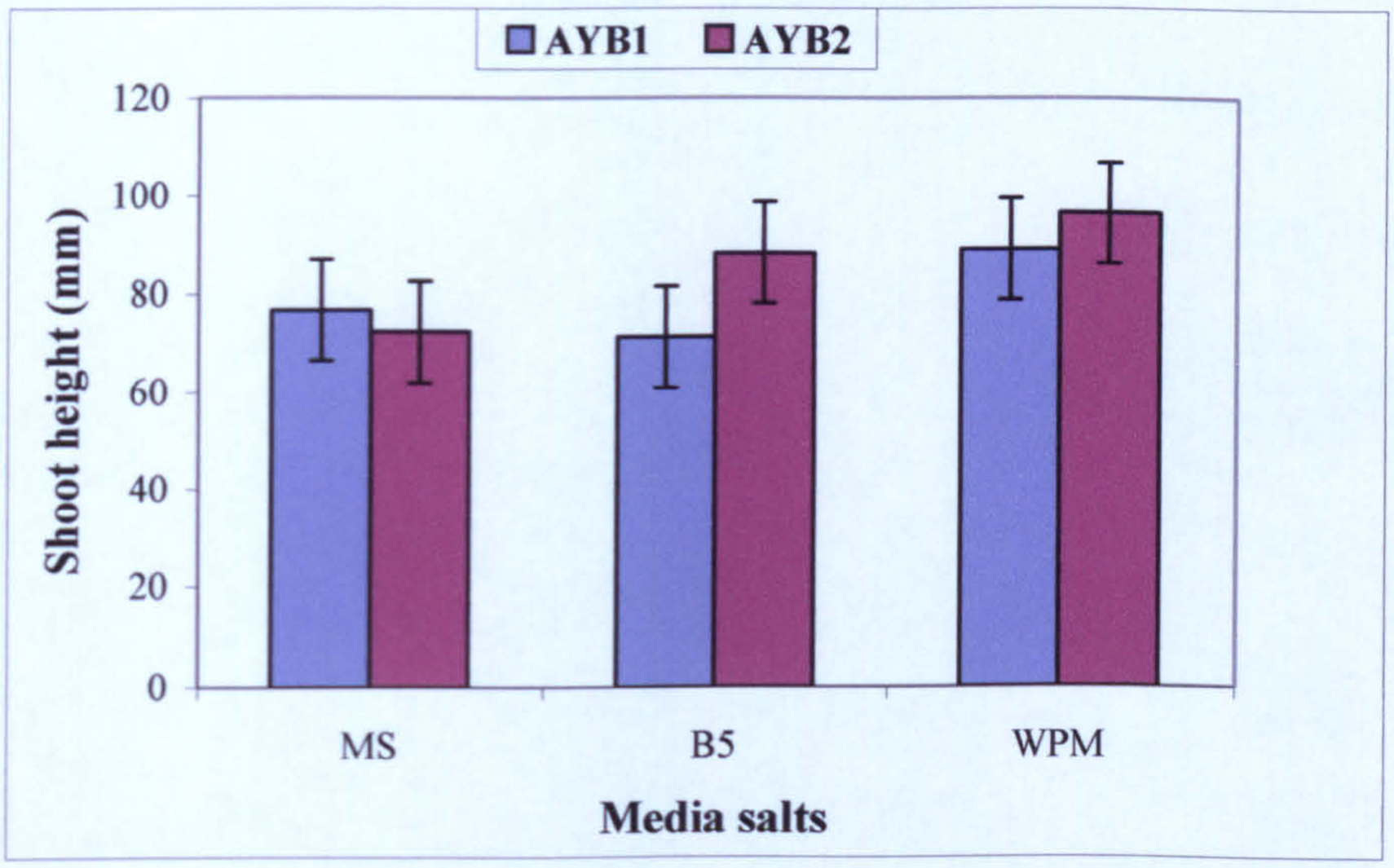

Figure 6.6 Effect of different media salts on mean shoot height of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=9) (Experiment 2)

Callus proliferation followed similar trends in both experiments with the largest size of callus [Experiment 1 ($F_{(2,36)}$ = 30.28, p<0.001) and Experiment 2 ($F_{(2,48)}$ = 16.07, p<0.001)] produced on MS medium (Figures 6.7 and 6.8). An effect of genotypic was observed only in Experiment 1, where AYB1 produced slightly more callus than AYB2 ($F_{(1,36)}$ = 5.54, p<0.05) (Figure 6.7). The interaction between media and landrace in Experiment 1 was detected because AYB1 produced more callus ($F_{(2,36)}$ = 4.38, p<0.05) on MS and B5 whereas AYB2 produced more on WPM (Figure 6.7). However, in Experiment 2, the interaction was detected because AYB1 produced more callus ($F_{(2,48)}$ = 3.68, p<0.05) than AYB2 on B5 medium (Figure 6.7). Culture medium was the only factor that had an influence on callus weight in both experiments [Experiment 1 ($F_{(2,36)}$ = 6.45, p<0.01) and Experiment 2 ($F_{(2,48)}$ = 29.88, p<0.001)] with MS and B5 being the most supportive and WPM the least (Tables 6.5 and 6.6).

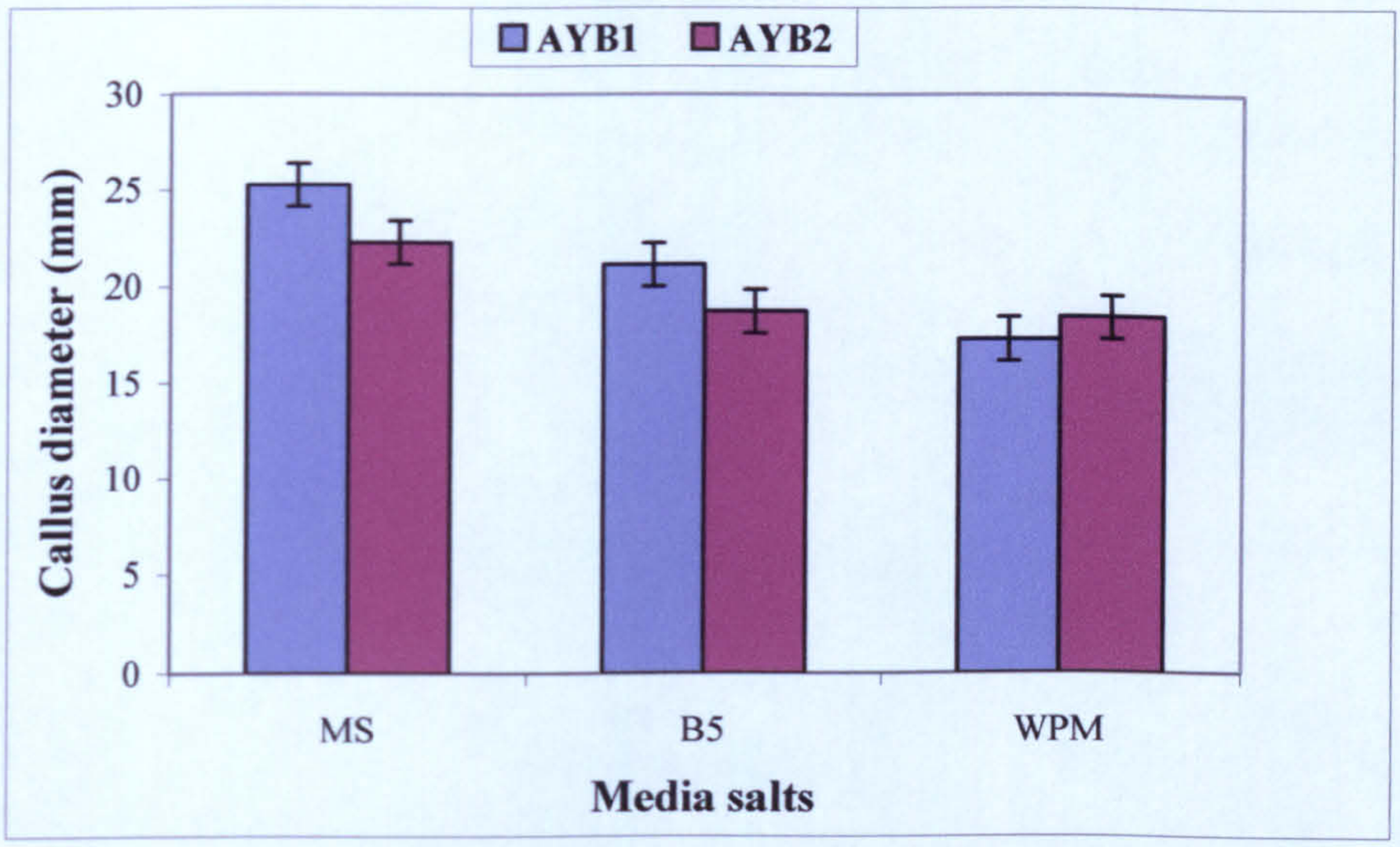

Figure 6.7 Effect of different media salts on mean size of callus (diameter) produced by AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=7) (Experiment 1)

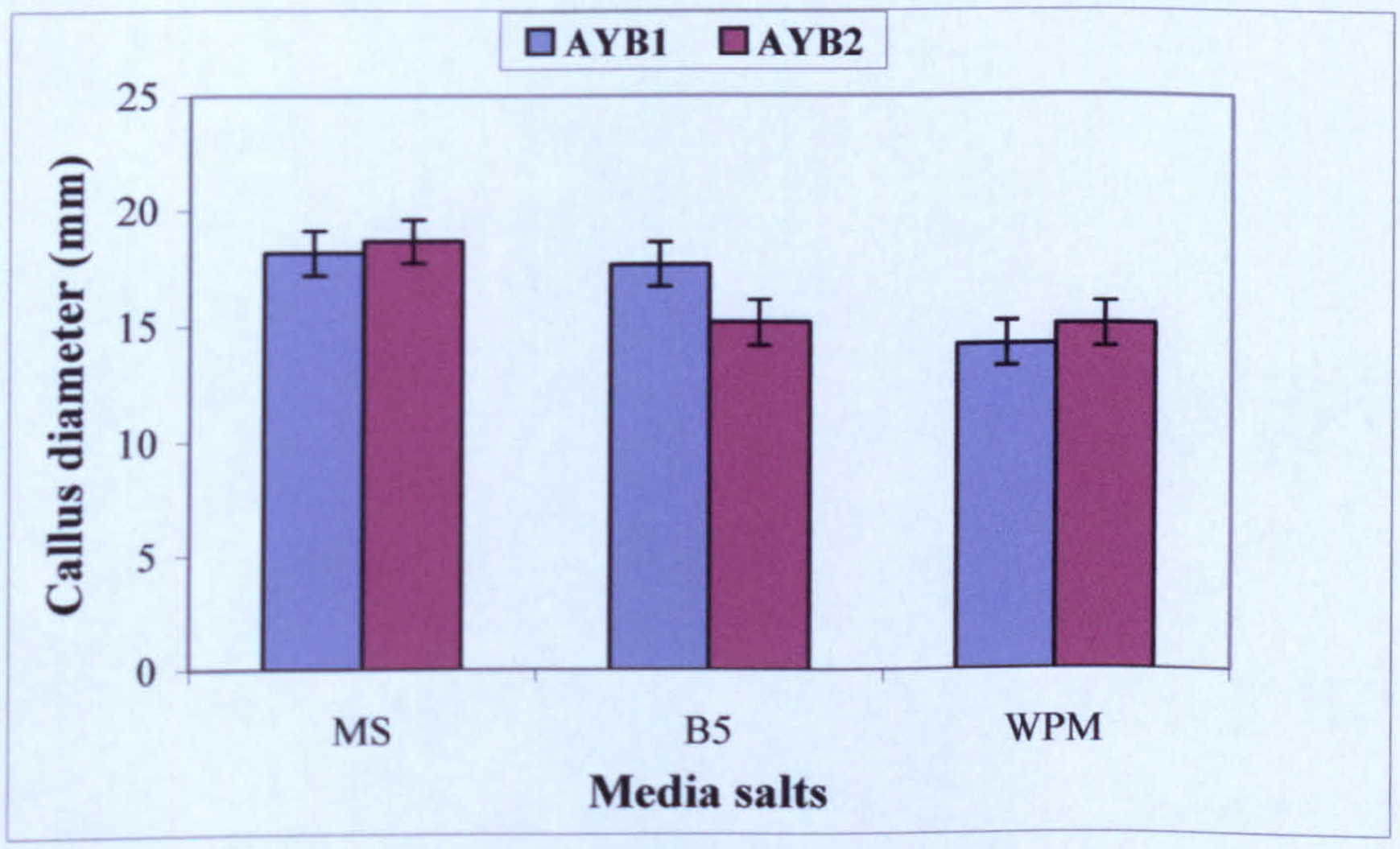

Figure 6.8 Effect of different media salts on mean callus size (diameter) produced by AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=9) (Experiment 2)

6.2.3.3 Discussion

A nutrient rich medium such as MS is essential to sustain the *in vitro* regeneration of AYB, as it is in other plants for the maximum growth for the duration of the culture without depletion of the nutrients (Jeong *et al.*, 1995). This confirms previous findings that MS is the most popular medium because most plants react favourably to it. Although both landraces reacted positively to MS, there was genotypic variation between them, and this supports the view of Pierik (1987) who stated that, whilst MS

is the most popular, it may not necessarily be optimal for growth and development of other plants because of its high salt content. WPM was found to be least beneficial to *in vitro* regeneration of AYB.

6.3 Shoot multiplication/proliferation from axenic explants

The aim is to produce more shoots by subculturing axenic shoots by either direct caulogenesis or axillary bud proliferation before caulogenesis.

6.3.1 Effect of cytokinins (BAP, TDZ and 2iP) on shoot multiplication

Cytokinin concentration of 0.25 mg and 0.5 l^{-1} were found to favour shoot growth compared to the other concentrations tried in Section 6.2. Therefore, these concentrations were used in this trial.

6.3.1.1 Materials and methods

Axenic cultures were initiated from nodal stem explants of AYB1 and AYB2 cultured for five weeks on MS medium containing 1.0 mg l^{-1} BAP for the production of explants for shoot multiplication. Nodal segments were excised from the axenic shoots and subcultured onto MS basal medium containing the cytokinins BAP, 2iP and TDZ at 0, 0.25 and 0.5 mg l^{-1}. The experiment was set up as a completely randomised design with 5 replicates (5 cultures each with one explant) per treatment. The cultures were incubated for 5 weeks under conditions described in Chapter 3, Section 3.7.1 after which callus size (diameter and weight), shoot number and shoot height were recorded.

6.3.1.2 Results

There were highly significant differences between landraces ($F_{(1,144)}$ = 27.66, p<0.001), type of cytokinin ($F_{(2,144)}$ = 18.81, p<0.001) and concentration of cytokinin ($F_{(2,144)}$ = 33.73, p<0.001) on stimulation of axillary shoot proliferation (Table 6.7). The interactions between landrace and type of cytokinin were significant ($F_{(2,144)}$ = 9.28, p<0.001) and between cytokinin and concentration ($F_{(4,144)}$ = 5.08, p<0.001) for shoot number. The difference in number of shoots between landraces was greater with TDZ than 2iP and BAP. More shoots were produced by AYB1 than AYB2 under all cytokinins with TDZ the most active and 2iP the least. There was no

difference in the weights of shoots of AYB1 and AYB2 but, shoots were relatively heavier ($F_{(2,142)}$ = 33.91, p<0.001) with 2iP than with TDZ and BAP (Table 6.8). Shoots possessed more nodes ($F_{(4,144)}$ = 6.61, p<0.001) on media containing cytokinin at any concentration than the control (Table 6.7). BAP produced more nodes and 2iP produced the least. Landraces also differed significantly ($F_{(1,144)}$ = 23.52, p<0.001) in the number of nodes produced with AYB2 possessing more nodes than AYB1.

The significant interaction between landrace, cytokinin and cytokinin concentration ($F_{(4,144)}$ = 6.69, p<0.001) showed AYB2 produced taller shoots with BAP and TDZ at 0.25 mg l^{-1} (Figure 6.9). There was no significant difference between landraces when all the cytokinins were at 0.5 mg l^{-1} (Figure 6.9). Media without cytokinin did not support any shoot elongation for AYB1. Shoot height produced with each cytokinin corresponded with number of nodes, i.e. BAP produced longer shoots that possessed more nodes than 2iP and TDZ.

Table 6.7 Effect of cytokinin and cytokinin concentration on mean shoot number and mean number of nodes of AYB1 and AYB2 landraces produced *in vitro* from axenic shoots. (n=5)

Factor	Shoot number						Number of nodes					
	AYB1			AYB2			AYB1			AYB2		
	BAP	2iP	TDZ	BAP	2iP	TDZ	BAP	2iP	TDZ	BAP	2iP	TDZ
Cytokinin concentration (mg l^{-1})												
0 (control)	0	0	0	0.4	0.4	0.4	0	0	0	0.6	0.6	0.6
0.25	1.4	0.4	1.1	1.9	0.4	2.8	2.0	0.6	1.2	3.2	0.7	2.7
0.50	1.2	0.6	0.7	1.6	0.6	2.7	2.2	1.0	0.9	2.2	1.4	2.0
s.e.d.	0.37						0.412					
d.f.	4						4					
Significance (0.05)												
Landrace	<0.001						<0.001					
Cytokinin	<0.001						<0.001					
Cytokinin concentration	<0.001						<0.001					
Landrace × cytokinin	<0.001						0.135					
Landrace × cytokinin concentration	<0.001						<0.001					
Cytokinin × cytokinin concentration	0.506						0.411					
Landrace × cytokinin × cytokinin concentration	0.051						0.236					

Table 6.8 Effect of cytokinin and cytokinin concentration on mean shoot weight and mean callus weight of AYB1 and AYB2 landraces produced *in vitro* from axenic shoots. (n=5)

Factor	**Shoot weight (g)**						**Callus weight (g)**					
	AYB1			AYB2			AYB1			AYB2		
	BAP	2iP	TDZ	BAP	2iP	TDZ	BAP	2iP	TDZ	BAP	2iP	TDZ
Cytokinin concentration (mg l^{-1})												
0 (control)	0	0	0	0.053	0.053	0.053	0	0	0	0.06	0.06	0.06
0.25	0.013	0.026	0.011	0.062	0.027	0.060	2.86	0.88	4.36	3.19	0.68	4.56
0.50	0.014	0.030	0.009	0.024	0.046	0.040	3.60	0.86	4.64	3.33	1.59	4.89
s.e.d.	0.0214						0.340					
d.f.	4						4					
Significance (0.05)												
Landrace	0.961						<0.001					
Cytokinin	<0.001						0.427					
Cytokinin concentration	0.706						<0.001					
Landrace × cytokinin	0.471						0.799					
Landrace × cytokinin concentration	0.689						<0.001					
Cytokinin × cytokinin concentration.	0.144						0.981					
Landrace × cytokinin × cytokinin conc.	0.676						0.203					

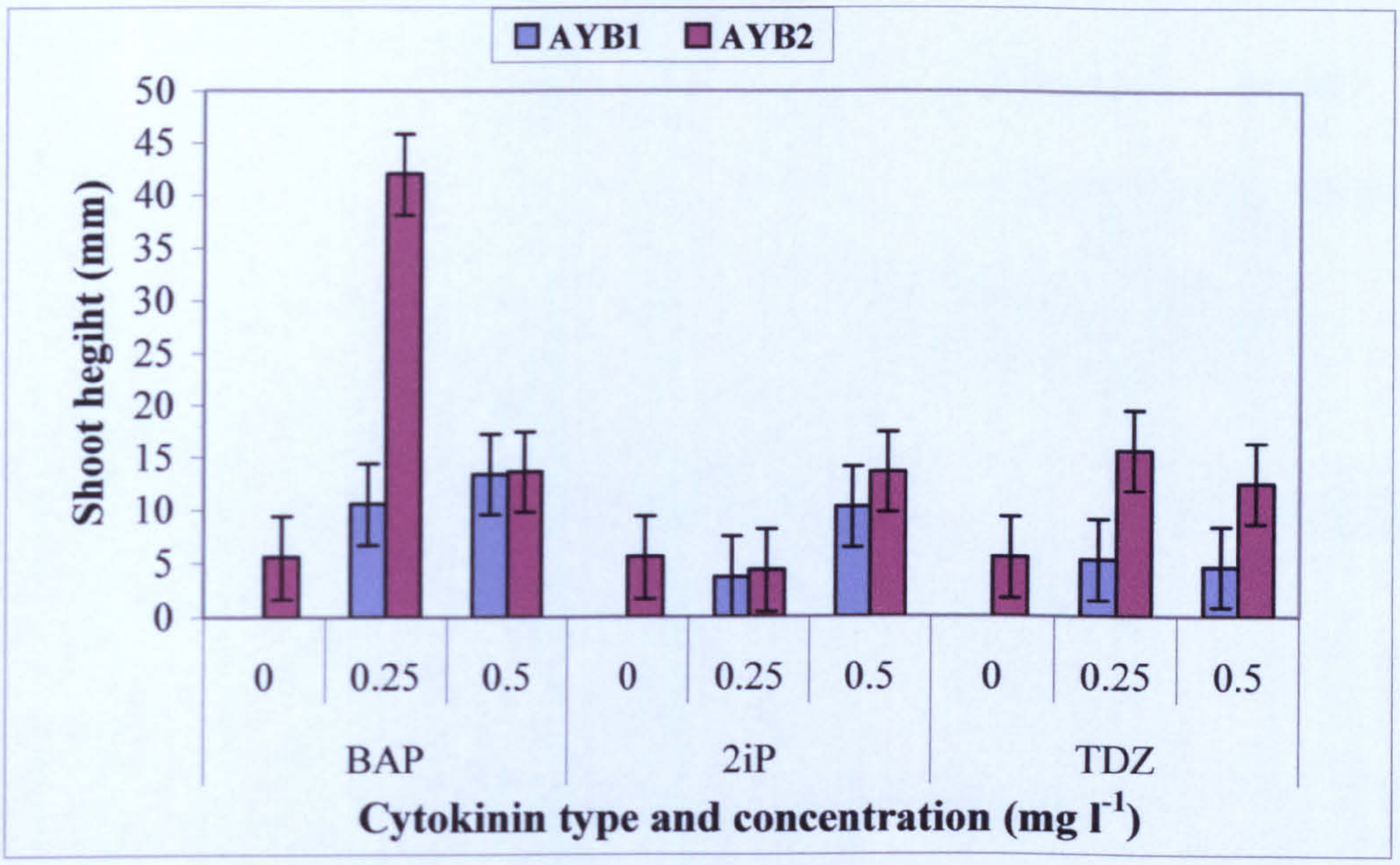

Figure 6.9 Effect of cytokinin type and concentration on mean shoot height of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5)

The addition of any of the three cytokinins to the culture medium significantly ($F_{(2,144)}$ = 33.91, p<0.001) increased the amount of callus produced, with TDZ the most active and 2iP the least (Figure 6.10). While there was no difference between the effect of the concentrations tested, there was a clear difference ($F_{(4,144)}$ = 12.77, p<0.001) between media with and without cytokinin (Figure 6.10). There was a significant ($F_{(2,144)}$ = 5.24, p<0.01) interaction between landrace and cytokinin concentration where AYB1 produced more callus than AYB2 at 0.5 mg l^{-1} but not at 0.25 mg l^{-1} (Figure 6.10). Differences in callus weight between the two landraces were also significant ($F_{(1,142)}$ = 145.92, p<0.001) with the heavier callus produced by AYB2 (Table 6.8). The strong interaction ($F_{(1,142)}$ = 36.81, p<0.001) between cytokinin and concentration showed that callus weight also depended on the type and concentration of cytokinin.

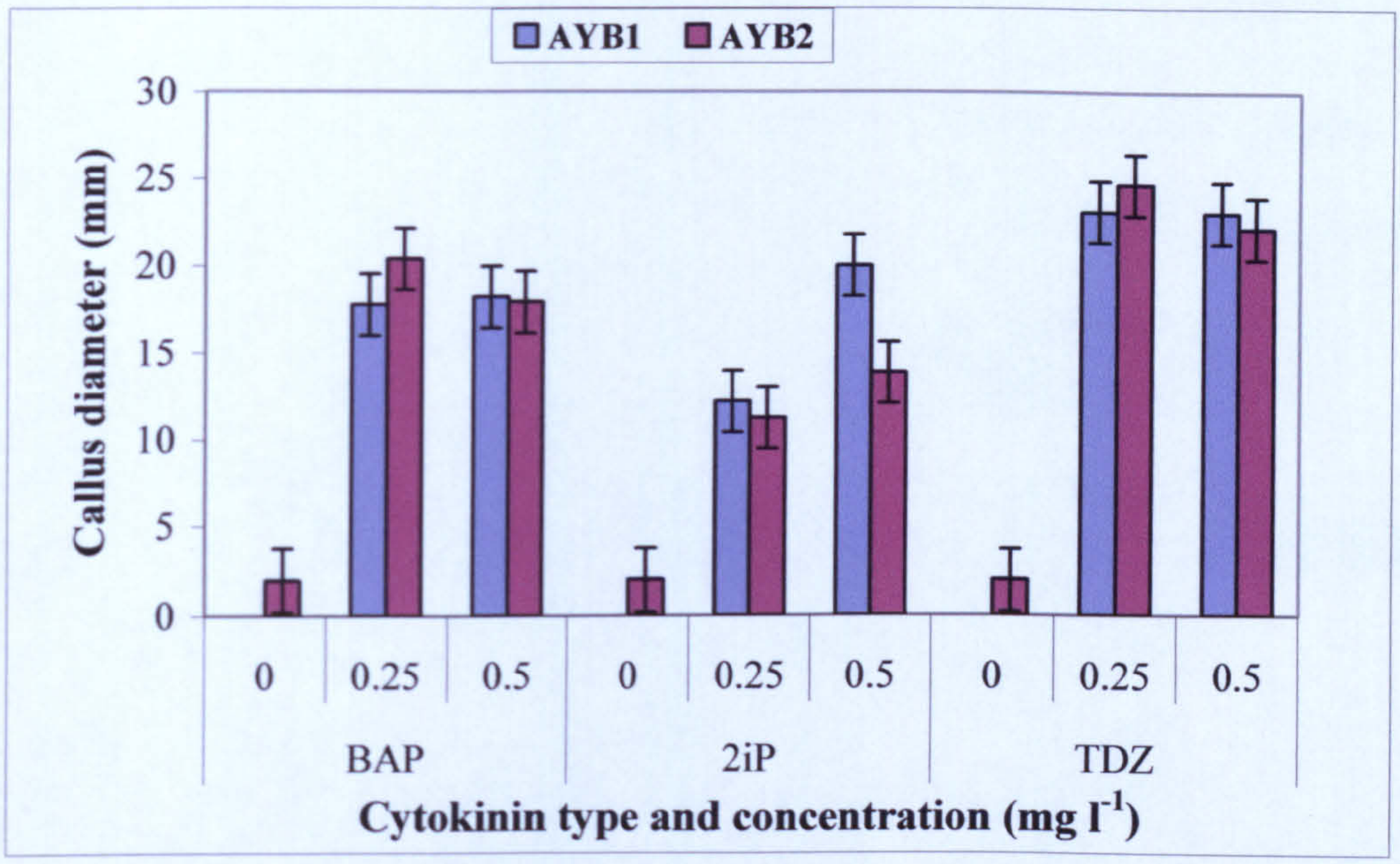

Figure 6.10 Effect of cytokinin type and concentration on mean callus size (diameter) produced by AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5)

6.3.1.3 Discussion

Thidiazuron, as observed in the experiments on the establishment of axenic cultures (Section 6.2.2), was a more active cytokinin than BAP and 2iP in terms of shoot multiplication and callus production confirming previous reports (Naik *et al.*, 1999). BAP was better for shoot growth/development although shoots were not long enough to be rooted. These shoots possessed more nodes than the shoots produced by other cytokinins, thereby conferring the benefit of shoot proliferation by subculturing of axenic nodal segments. Both landraces produced large amounts of callus (size and weight) with more produced in the presence of cytokinin, confirming similar observations made by other researchers on black gram (*Vigna mungo*) (Geetha *et al.*, 1997b) and adzuki bean (*Vigna angularis*) (Avenido and Hattori, 2000).

6.3.2 Effect of auxin polar transport inhibitor on *in vitro* regeneration from axenic nodal explants

It has been suggested that addition of auxin polar transport inhibitors, 2,3,5-triiodobenzoic acid (TIBA) and *N*-(1-naphthyl)phtalamic acid (NPA), to regeneration media may inhibit the transport of endogenous IAA to regeneration sites, so that an auxin/cytokinin balance becomes more favourable for the regeneration of shoot buds (Charriere and Hahne, 1998; Nakano *et al.*, 2000). Replacement of somatic embryos

by caulogenesis from immature zygotic embryos of sunflower progressed with increase in concentration of NPA (10-70 μM) and TIBA (10-100 μM) (Charriere and Hahne, 1998). Nakano *et al.* (2000) restored regeneration potential of 75-months-old cultures to about 10-fold increases in the number of regenerated shoot buds through addition of 0.5 or 5 μM TIBA in combination with 5 μM BAP or 5 μM TDZ. Two experiments were carried out to test the hypothesis that TIBA will induce shoots formation instead of callus proliferation from axenic explants of AYB. Due to shortage of AYB2 material, only AYB1 landrace was used in Experiment 1. In Experiment 2, both landraces were used.

6.3.2.1 Materials and methods

Axenic cultures were established on MS medium containing 1.0 mg l^{-1} BAP as described in Section 6.2.1.1. Nodal explants of AYB1 were excised and subcultured onto the same medium as used in the establishment of axenic cultures but also enriched with TIBA at 0.0 (control), 0.5, 1.5 and 2.5 mg l^{-1} in Experiment 1. In Experiment 2, nodal explants of AYB1 and AYB2 were subcultured onto the same media as above but enriched with TIBA at 0.0 (control), 2.5, 5.0 and 10 mg l^{-1}. Each experiment was set up as a completely randomised design with 5 replicates (5 culture vessels each with one explant) per treatment. The cultures were incubated for five weeks (as described in Chapter 3, Section 3.7.1) after which data was recorded for shoot growth and callus production.

6.3.2.2 Results

Addition of TIBA to the culture medium was not beneficial for the number of shoots produced or their growth (weight) in the two experiments (Tables 6.9 and 6.10). AYB2 produced more shoots ($F_{(1,24)} = 11.31$, $p<0.01$) than AYB1 (Table 6.10). In Experiment 1, the higher TIBA concentrations (1.5 and 2.5 mg l^{-1}) resulted in shorter shoots ($F_{(3,16)} = 4.47$, $p<0.05$) (Figure 6.11). A similar trend was observed in Experiment 2 but the differences in shoot height were not significant (Figure 6.12). The weight of shoots was also not affected by treatments in both experiments (Tables 6.9 and 6.10). Adventitious shoot proliferation was not observed and all the shoots grew from axillary buds.

Table 6.9 Effect of TIBA on mean shoot number, mean shoot weight and mean callus weight produced by AYB1 landrace. (n=5) (Experiment 1)

Factor	Shoot number	Shoot weight (g)	Callus weight (g)
TIBA concentration			
0	1.4	0.007	4.96
0.5	1.0	0.012	5.59
1.5	1.0	0.030	3.15
2.5	1.2	0.008	2.46
s.e.d.	0.22	0.0167	0.532
d.f.	3	3	3
Significance (0.05)	0.261	0.493	<0.001

Table 6.10 Effect of TIBA on mean shoot number, mean shoot weight and mean callus weight produced by AYB1 and AYB2 landraces. (n=5) (Experiment 2)

Factor	Shoot number		Shoot weight (g)		Callus weight (g)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
TIBA concentration ($mg\ l^{-1}$)						
0	1.0	3.5	0.034	0.063	0.90	1.88
2.5	1.3	1.8	0.024	0.019	1.08	2.28
5.0	2.1	2.5	0.035	0.038	0.90	1.80
10.0	1.8	2.5	0.016	0.042	0.25	1.05
s.e.d.	0.78		0.0193		0.551	
d.f.	3		3		3	
Significance (0.05)						
Landrace	0.003		0.285		0.002	
TIBA	0.518		0.180		0.082	
Landrace × TIBA conc.	0.290		0.535		0.961	

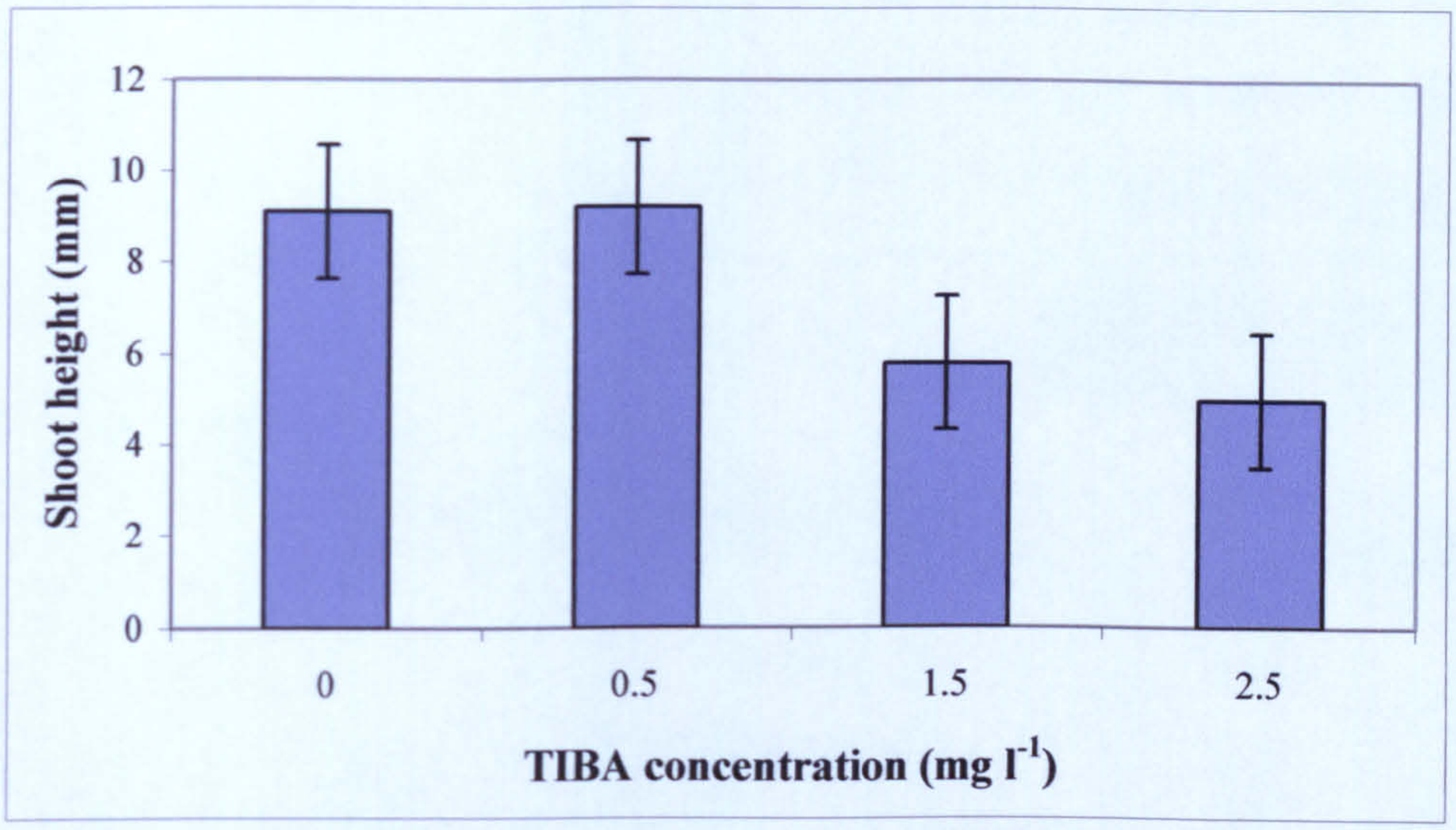

Figure 6.11 Effect of TIBA on mean shoot height of AYB1 landrace. Error bars = 2 *s.e.d.* (n=5) (Experiment 1)

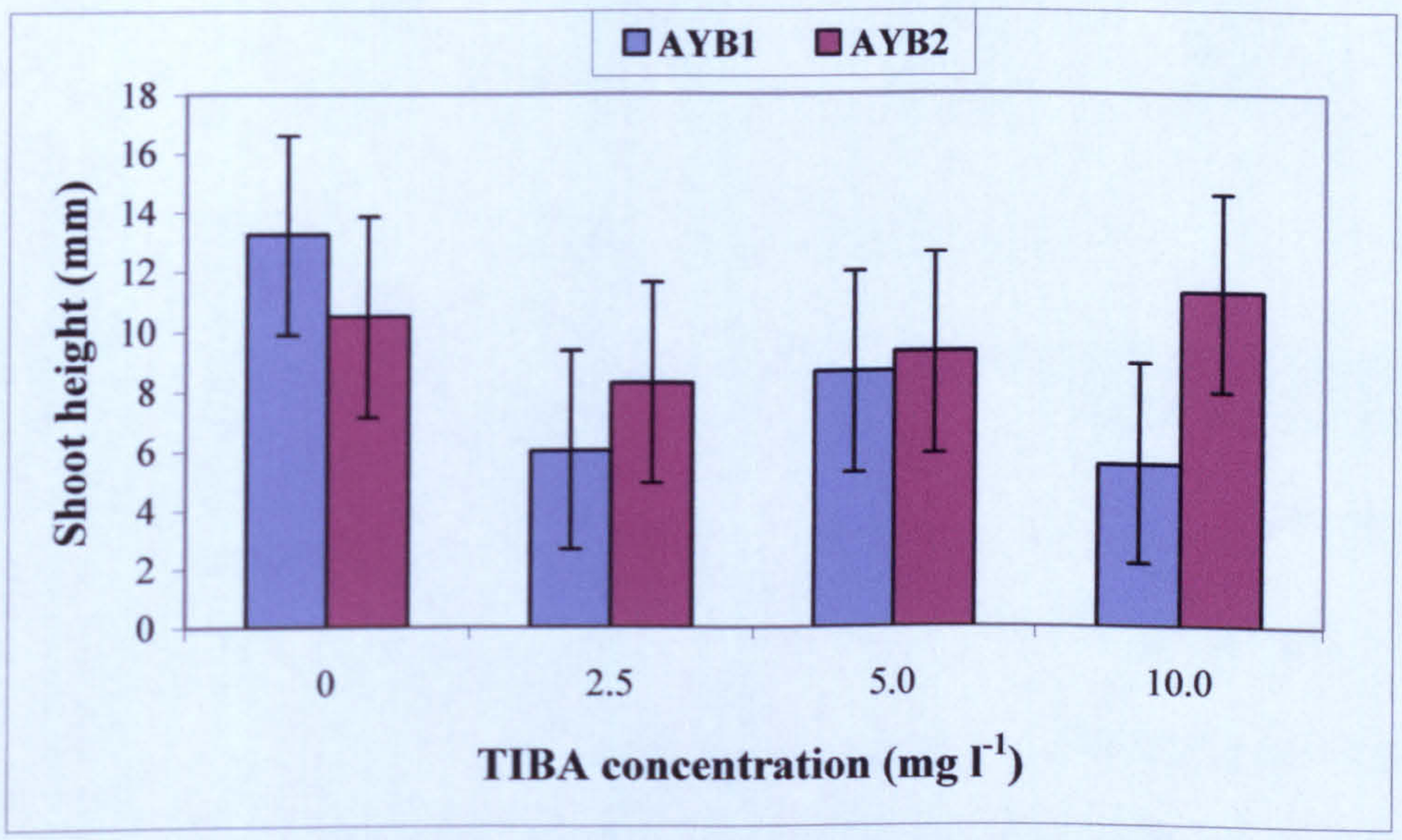

Figure 6.12 Effect of TIBA on mean shoot height of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5) (Experiment 2)

All the cultures produced excessive amounts of callus (Tables 6.9 and 6.10, Figures 13 and 14). In the first experiment, differences in the amounts (size) of callus between TIBA concentrations were significant ($F_{(3,16)} = 13.70$, $p<0.001$) with 2.5 mg l^{-1} giving less callus, whereas in Experiment 2, although the trend was similar, the difference was not significant (Figures 13 and 14). The highest level of TIBA (10.0 mg l^{-1}) did not significantly reduce callus growth except between the landraces where AYB2 produced more callus ($F_{(1,16)} = 7.23$, $p<0.05$) than AYB1. Callus weight

differed significantly ($F_{(3,16)}$ = 15.37, p<0.001) between concentrations in Experiment 1 with control media and media containing 0.5 mg l^{-1} producing the heavier callus (Table 6.9). In Experiment 2, there was no differences in the weight of callus produced on different TIBA concentrations, but the landraces differed significantly ($F_{(2,24)}$ = 12.39, p<0.01) with AYB2 producing more callus biomass (Table 6.10).

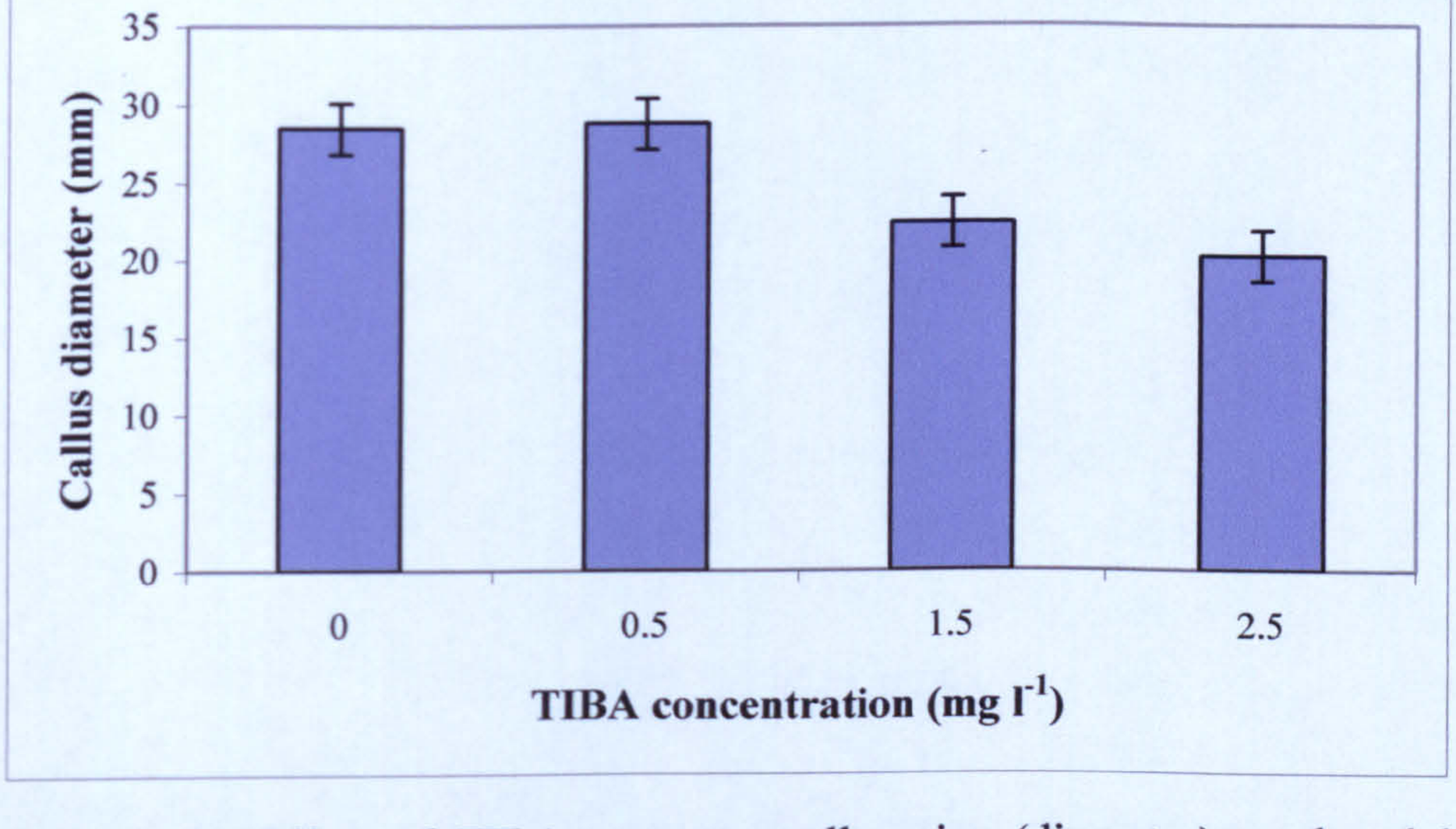

Figure 6.13 Effect of TIBA on mean callus size (diameter) produced by AYB1 landrace. Error bars = 2 *s.e.d.* (n=5) (Experiment 1)

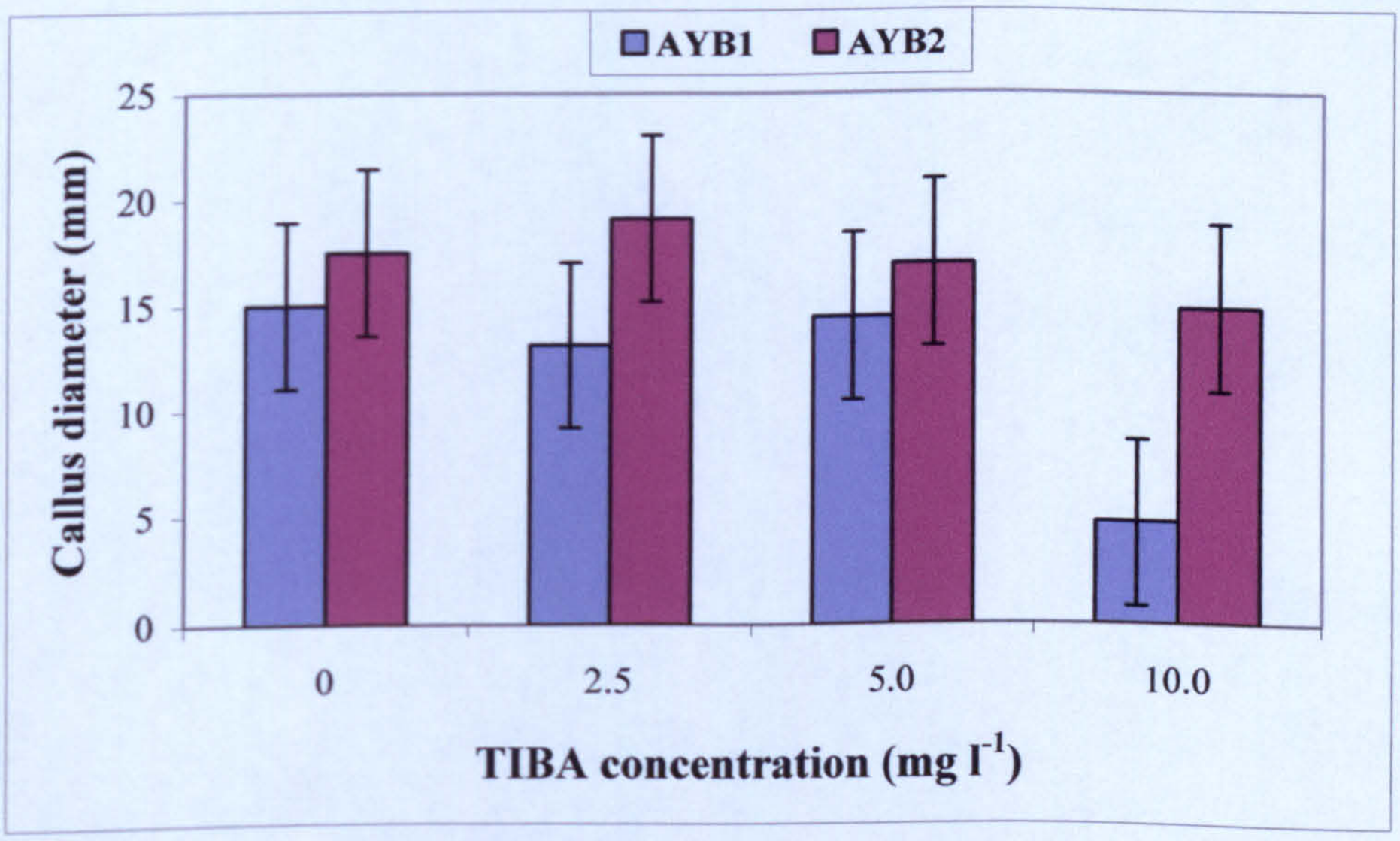

Figure 6.14 Effect of TIBA on mean callus size (diameter) produced by AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5) (Experiment 2)

6.3.2.3 Discussion

In both experiments, the cultures produced large amounts of callus with little proliferation and development of shoots. Contrary to the original hypothesis, TIBA did not reduce the amount of callus produced, however, the literature reports that the effect of TIBA on shoot cultures tends to be species dependent. Voyiatzi *et al.* (1995) reported reduced apical dominance and increased axillary branching of hybrid tea rose cv. 'Dr. Verbage' and that this successfully replaced conventional manual tipping or shoot tip pinching. Orlikowska *et al.* (2000) observed that shoot tip removal and additional defoliation improved axillary branching of croton/garden croton (*Codiaeum variegatum* Blume *var. pictum* Muell. Arg.) while addition of TIBA did not replace defoliation nor diminish the size of callus formed at the base of shoots.

6.3.3 Effect of media on shoot proliferation and growth

MS medium is the most popular as most plants react favourably to it, but its content of salt is too high for some plants (Pierik, 1987). It was also found in Section 6.2.3 to be more suitable for AYB than the other media salts although the cultures were still accompanied by excessive callus proliferation. Since it is not entirely optimal for all plant species, adjustments are sometimes imposed or an alternative formulation used. Ohki and Sawaki (1999) established the benefit to shoot proliferation of *Delphinium cardinale* by adjusting the strength of MS medium by one third. Chaturvedi *et al.* (2004) reported that half strength MS medium required initially to establish nodal segment cultures of neem tree (*Azadirachta indica*) also stimulated subsequent multiple shoot formation when enriched with BAP (1μM) + GA_3 (0.5 μM) or casein hydrolysate (CH) (250 mg l^{-1}). An experiment was carried out to test the hypothesis that adjustment of strength of MS will induce shoot proliferation with minimum callus production.

6.3.3.1 Materials and methods

Nodal segments of AYB1 and AYB2 landraces were excised from axenic shoot cultures established (as described in Section 6.2.1) on MS medium containing 1.0 mg l^{-1} BAP and subcultured on the following MS media: half strength MS without BAP, full strength MS medium without BAP, half strength MS plus BAP and full strength MS medium plus 0.5 mg l^{-1} BAP determined in Section 6.2.1 to be one of the

suitable concentration for shoot regeneration. The experiment was set up as a completely randomised design with 9 replicates (9 culture vessels each with one explant) per treatment. The cultures were incubated for five weeks under conditions described in Chapter 3, Section 3.7.1 after which data were recorded for shoot and callus growth.

6.3.3.2 Results

Shoot formation and growth of shoots were highest with full strength MS medium containing BAP (Table 6.11). There was an interaction ($F_{(3,64)} = 4.02$, $p<0.05$) between media and landrace with AYB1 responding best to full strength MS as the number of shoots was high with or without BAP. However, AYB2 responded negatively to MS but positive to BAP as it produced about 2 or more shoots on media containing BAP. This same trend was observed for shoot elongation (height) and number of nodes. The number of nodes produced did not differ between landraces but was more dependent on the medium ($F_{(3,64)} = 11.27$, $p<0.001$) and full strength MS medium containing BAP produced the highest number (Table 6.11). The interaction between landrace and medium on shoot height was significant ($F_{(3,64)} = 3.67$, $p<0.05$) (Figure 6.15). While shoot height differed between landraces ($F_{(1,64)} = 5.43$, $p<0.05$) with AYB2 producing the tallest, it was more influenced by medium ($F_{(3,64)} = 23.03$, $p<0.001$) with full strength medium containing cytokinins producing the tallest shoots (Figure 6.15). The trend in origin of shoots was similar to that in the previous sections where shoots came from axillary buds.

Table 6.11 Effect of media on mean shoot number, mean number of nodes and mean callus weight of AYB1 and AYB2 landraces. (n= 9)

Factor	**Shoot number**		**Number of nodes)**		**Callus weight (g)**	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Media						
½MS	0.7	1.0	0.8	0.8	0.06	0.01
MS	1.8	0.3	0.8	1.2	0.11	0
½MS + BAP	0.7	1.9	1.1	1.4	0.80	1.97
MS + BAP	1.8	2.6	3.3	2.7	4.46	5.01
s.e.d.	0.36		0.59		0.345	
d.f.	3		3		3	
Significance (0.05)						
Landrace	0.010		0.925		0.026	
Media	<0.001		<0.001		<0.001	
Landrace × Media.	0.011		0.587		0.035	

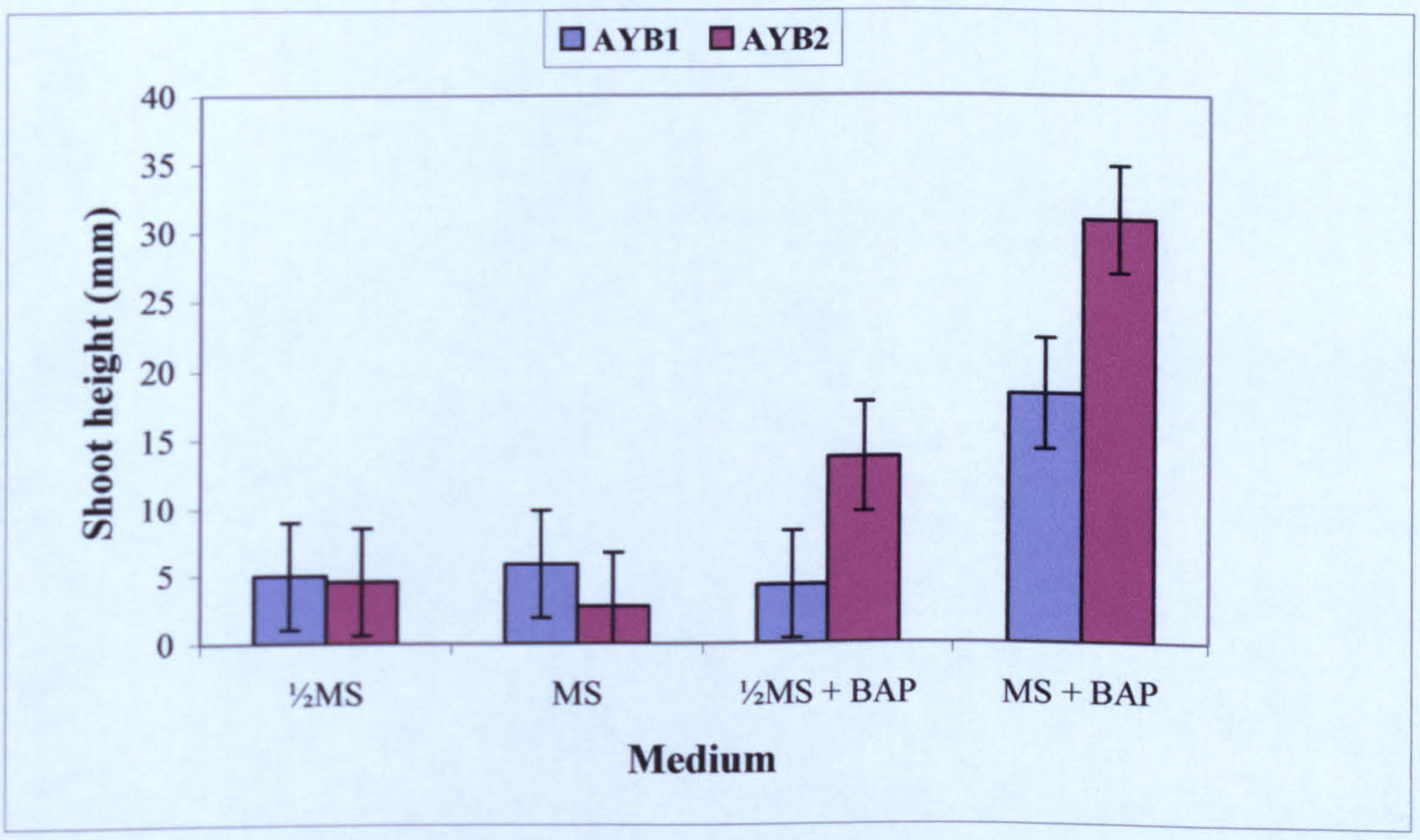

Figure 6.15 Effect of media on mean height of shoots generated from axenic explants of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=9)

Explants cultured on the media without cytokinins produced very little or no callus (Table 6.11 and Figure 6.16). The interaction ($F_{(3,64)} = 8.74$, $p<0.001$) between landrace and medium indicates that AYB2 produced more callus than AYB1 on half strength MS containing BAP while AYB1 produced more on full strength MS without cytokinins. The largest amount of callus (diameter) for both landraces was

produced on full MS containing BAP. The trend was similar for callus weight (Table 6.11).

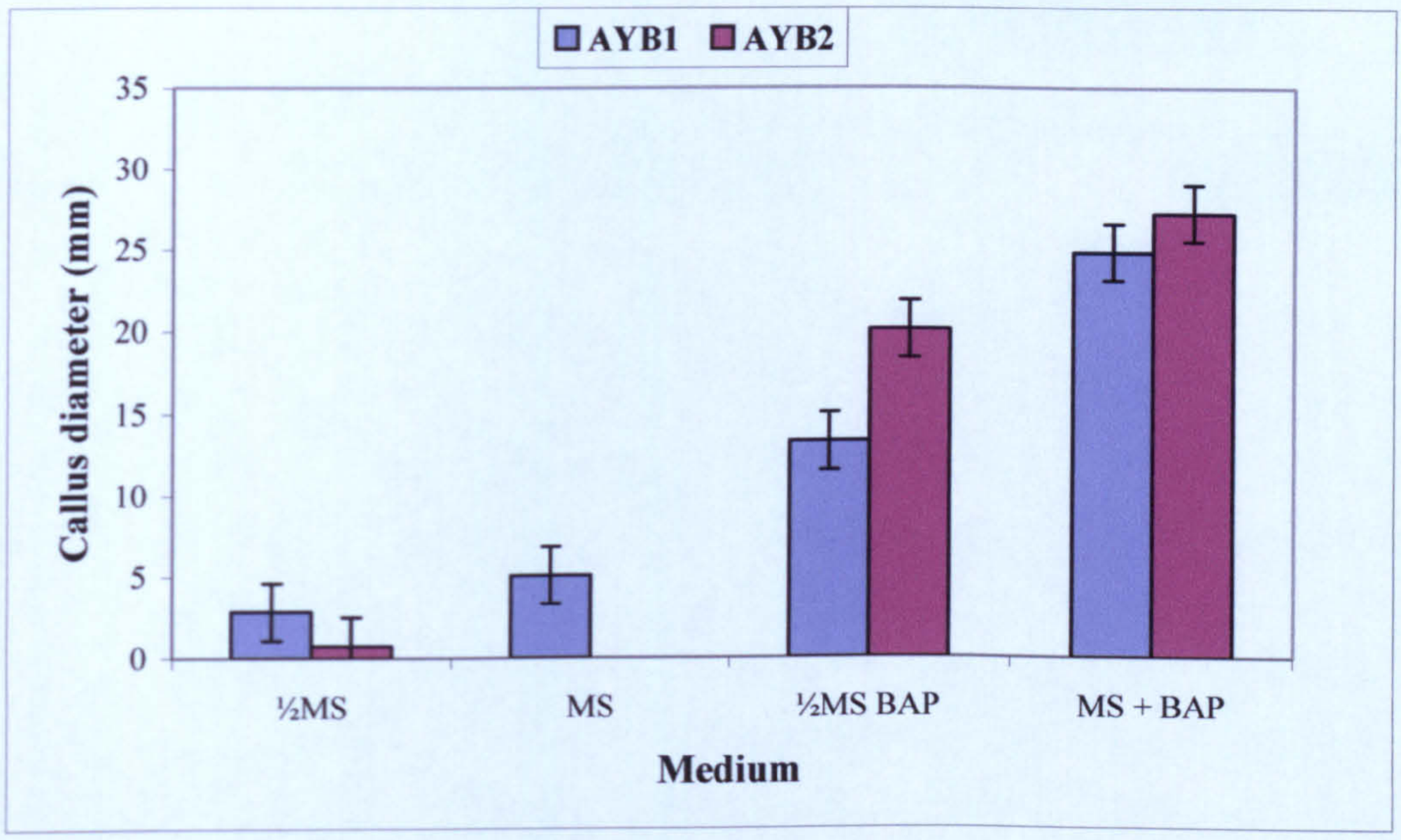

Figure 6.16 Effect of media on mean size of callus (diameter) produced by axenic explants of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=9)

6.3.3.3 Discussion

AYB requires MS medium at full strength for reliable proliferation and growth/development of shoots. However, this regeneration capacity is also accompanied by the production of excessive callus. This contrasts with findings for black gram (*Vigna mungo*), where half strength MS produced both callus and shoot buds while full strength MS inhibited bud formation (Das *et al.*, 1998). Likewise, Ohki and Sawaki (1999) established that adjusting MS strength by one third benefited shoot proliferation of scarlet larkspur (*Delphinium cardinale*). Chaturvedi *et al.* (2004) reported that half strength MS enriched with BAP and GA_3 or casein hydrolysate stimulated multiple shoot formation, but shoot growth was not supported and the shoots remained compact and stunted until MS was used at full strength. This observation is consistent with the present study and suggests there may be differences between species. The better shoot growth of AYB on full strength MS observed in this experiment supports findings from the comparison between MS, WPM and B5 salts (Section 6.2.3).

The need for cytokinin in the *in vitro* regeneration of AYB has also been confirmed in this experiment. All growth parameters were affected by absence of BAP in the media. Although full strength MS was found to be favourable for AYB regeneration, shoot growth and callus growth were highly influenced by BAP. This observation is consistent with the literature and findings reported in Section 6.2.1 of the current study.

6.3.4 Effect of GA_3 on shoot proliferation and elongation

Synergistic effect of BAP and GA_3 on *in vitro* shoot growth from nodal explants has been reported by many researchers (Pattnaik and Chand, 1997; Purohit and Singhvi, 1998; Chitra and Padmaja 1999; Vengadesan *et al.*, 2002). Purohit and Singhvi (1998) reported improved shoot elongation and an enhanced rate of shoot multiplication of *Achras sapota* (L.) when GA_3 (1 mg l^{-1}) was incorporated in the medium during the first subculture after establishment. Pattnaik and Chand (1997) achieved faster bud break both in apical shoots and nodal explants and an enhanced frequency of bud break in three mulberries species through the incorporation of GA_3 (0.2-0.4 mg l^{-1}) along with BAP (1.0 mg l^{-1}). Three experiments were carried out to test the hypothesis that gibberellin will encourage shoot elongation and suppresses callus proliferation of AYB axenic explants.

6.3.4.1 Materials and methods

Axenic cultures of AYB1 and AYB2 landraces were established on MS medium containing 1.0 mg l^{-1} BAP for five weeks under conditions described in Chapter 3, Section 3.7.1. Thereafter, nodal segments were excised and subcultured on MS medium containing BAP and GA_3 as described in Tables 6.12, 6.13 and 6.14. These cultures were also incubated for five weeks under conditions described in Chapter 3, Section 3.7.1 after which shoot growth and callus production data were recorded. Experiments 1 and 3 had 8 replicates (cultures vessels each containing 1 explant) while Experiment 2 had 7 replicates.

Table 6.12 Experiment 1 treatments.

BAP concentration (mg l^{-1})	GA_3 concentration (mg l^{-1})		
	0	0.25	0.5
0	0	-	0.5
0.25	-	0.25+0.25	-
0.50	0.5	-	-

Table 6.13 Experiment 2 treatments.

BAP concentration (mg l^{-1})	GA_3 concentration (mg l^{-1})		
	0	0.25	0.5
0	-	0.25	0.25
0.5	0.5	0.5+0.25	0.5+0.5
2.5	2.5	2.5+0.25	2.5+0.5

Table 6.14 Experiment 2 treatments.

BAP concentration (mg l^{-1})	GA_3 concentration (mg l^{-1})				
	0	0.5	1.5	2.5	5.0
0.5	0.5+0	0.5+0.5	0.5+1.5	0.5+2.5	0.5+2.5
2.5	2.5+0	2.5+0.5	2.5+1.0	2.5+2.5	2.5+5.0

6.3.4.2 Results

As in other experiments, shoots originated from axillary buds rather adventitious growth. In Experiment 1, the combination of BAP and GA_3 produced the best growth with more shoots ($F_{(3,56)} = 23.37$, $p<0.001$) compared to the other media (Table 6.15). The control medium (without any PGRs) was the least effective. In Experiment 2, with higher concentrations of BAP and GA_3, differences in shoot number were only significant ($F_{(7,80)} = 4.76$, $p<0.001$) where GA_3 was used alone (Table 6.16). In Experiment 3, when GA_3 was not used alone, there were no differences between the treatments in the number of shoots produced, however, differences between the landraces were significant ($F_{(1,139)} = 17.43$, $p<0.001$) with AYB2 producing more shoots than AYB1 (Table 6.17).

Shoot weight was not affected by media formulation in Experiments 2 and 3. In Experiment 2, the interaction between landrace and media formulation was significant ($F_{(7,80)} = 4.36$, $p<0.001$) (Table 6.16). Shoot weight differed ($F_{(1,139)} = 36.34$, $p<0.001$) between landraces in Experiment 3 with AYB2 producing the heaviest shoots (Table 6.17). The number of nodes produced corresponded to shoot

height for Experiments 1 and 2 but not in Experiment 3 where the only difference was between landraces ($F_{(1,139)} = 21.15, p<0.001$) (Table 6.17).

Shoot height varied between the media treatments in Experiments 1 ($F_{(3,56)} = 27.98$, $p<0.001$), 2 ($F_{(7,80)} = 7.16, p<0.001$) and 3 ($F_{(9,139)} = 2.00$ $p<0.001$) and the longest shoots were produced with equal concentrations of BAP and GA_3 (0.25 mg l^{-1} for Experiment 1) and (0.5 mg l^{-1} for Experiments 2 and 3) (Figures 6.17, 6.18 and 6.19). This trend was not observed when equal concentrations increased to 2.5 mg l^{-1} in Experiment 3. Landraces differed ($F_{(1,139)} = 24.72, p<0.001$) with AYB2 producing the taller shoots than AYB1 particularly in relation to media where GA_3 was used alone (Figure 6.17).

Table 6.15 Effect of GA_3 on mean shoot number, mean number of nodes and mean callus weight of AYB1 and AYB2 landraces. (n=8) (Experiment 1)

Factor	**Shoot number**		**Number of nodes**		**Callus weight (g)**	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Treatment (PGR mg l^{-1})						
0 (control)	0	0.5	0	0.6	0.01	0.07
0.5 (GA_3)	0.3	0.9	0.5	1.0	0	0.18
0.5 (BAP)	1.4	1.5	2.5	2.1	3.61	3.33
0.25 + 0.25 (BAP + GA_3)	2.3	2.3	3.0	2.9	3.72	3.96
s.e.d.	0.37		0.41		0.418	
d.f.	3		3		3	
Significance (0.05)						
Landrace	0.100		0.449		0.808	
Treatment	<0.001		<0.001		<0.001	
Landrace × treatment.	0.596		0.256		0.821	

Table 6.16 Effect of GA on mean shoot number, mean shoot weight, mean number nodes and mean callus weight of AYB1 and AYB2 landraces. (n=7) (Experiment 2)

Factor	Shoot number		Shoot weight (g)		Number of nodes		Callus weight (g)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Treatment (PGR mg l^{-1})								
0 5 (BAP)	1.8	1.5	0.034	0.040	2.7	1.8	4.16	3.88
2.5 (BAP)	1.0	1.5	0.005	0.040	1.0	0.8	3.30	3.13
0.5 + 0.25 (BAP + GA_3)	2.2	0.7	0.034	0.010	4.0	0.7	3.98	4.22
2.5 + 0.25 (BAP + GA_3)	1.3	1.2	0.019	0.018	2.0	1.3	3.86	4.23
0.5 + 0.5 (BAP + GA_3)	1.3	2.3	0.023	0.061	3.2	3.5	4.09	4.78
2.5 + 0.5 (BAP + GA_3)	1.8	1.7	0.014	0.052	1.2	3.7	3.94	4.01
0.25 (GA_3)	0.3	0	0.026	0	0.3	0	0.03	0
0.5 (GA_3)	0.2	0.3	0.016	0.001	0.8	0.2	0.05	0.03
s.e.d.	0.62		0.0117		0.95		0.540	
d.f.	7		7		7		7	
Significance (0.05)								
Landrace	0.636		0.278		0.243		0.561	
Treatment	<0.001		0.062		<0.001		<0.001	
Landrace × treatment	0.226		0.014		0.011		0.932	

Table 6.17 Effect of GA_3 on mean shoot number, mean shoot weight, mean number of nodes and mean callus weight of AYB1 and AYB2 landraces. (n=8) (Experiment 3)

Factor	Shoot number		Shoot weight (g)		Number of nodes		Callus weight (g)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Treatment (PGR mg l^{-1})								
0 5 (BAP)	1.4	1.5	0.013	0.028	1.9	2.0	2.69	3.14
2.5 (BAP)	0.6	1.6	0.008	0.030	1.1	2.6	1.50	2.85
0.5 + 0.5 (BAP + GA_3)	1.1	1.6	0.023	0.037	1.8	2.6	3.54	4.29
0.5 + 1.0 (BAP + GA_3)	1.4	1.1	0.011	0.035	1.6	2.4	1.56	3.17
0.5 + 2.5 (BAP + GA_3)	1.1	1.5	0.015	0.032	1.9	2.9	2.36	2.43
0.5 + 5.0 (BAP + GA_3)	1.0	1.5	0.018	0.030	2.3	2.9	2.78	2.71
2.5 + 0.5 (BAP + GA_3)	0.8	1.4	0.015	0.029	1.1	3.0	1.74	2.56
2.5 + 1.0 (BAP + GA_3)	0.9	1.6	0.016	0.049	1.3	3.5	1.74	2.35
2.5 + 2.5 (BAP + GA_3)	0.4	1.1	0.005	0.020	0.6	1.4	1.09	2.04
2.5 + 5.0 (BAP + GA_3)	0.9	1.4	0.012	0.024	1.4	2.4	1.68	2.51
s.e.d.	0.37		0.0094		0.74		0.600	
d.f.	9		9		9		9	
Significance (0.05)								
Landrace	<0.001		<0.001		<0.001		<0.001	
Treatment	0.369		0.141		0.213		<0.001	
Landrace × treatment	0.502		0.862		0.685		0.672	

Plate 6.3 Effect of combining of GA_3 and BAP on *in vitro* regeneration. T1 (0 mg l^{-1} PGRs/control), T2 (0.5 mg l^{-1} GA_3), T3 (0.5 mg l^{-1} BAP) and T4 (0.25 BAP +0.25 mg l^{-1} GA_3). Bar = 20 mm.

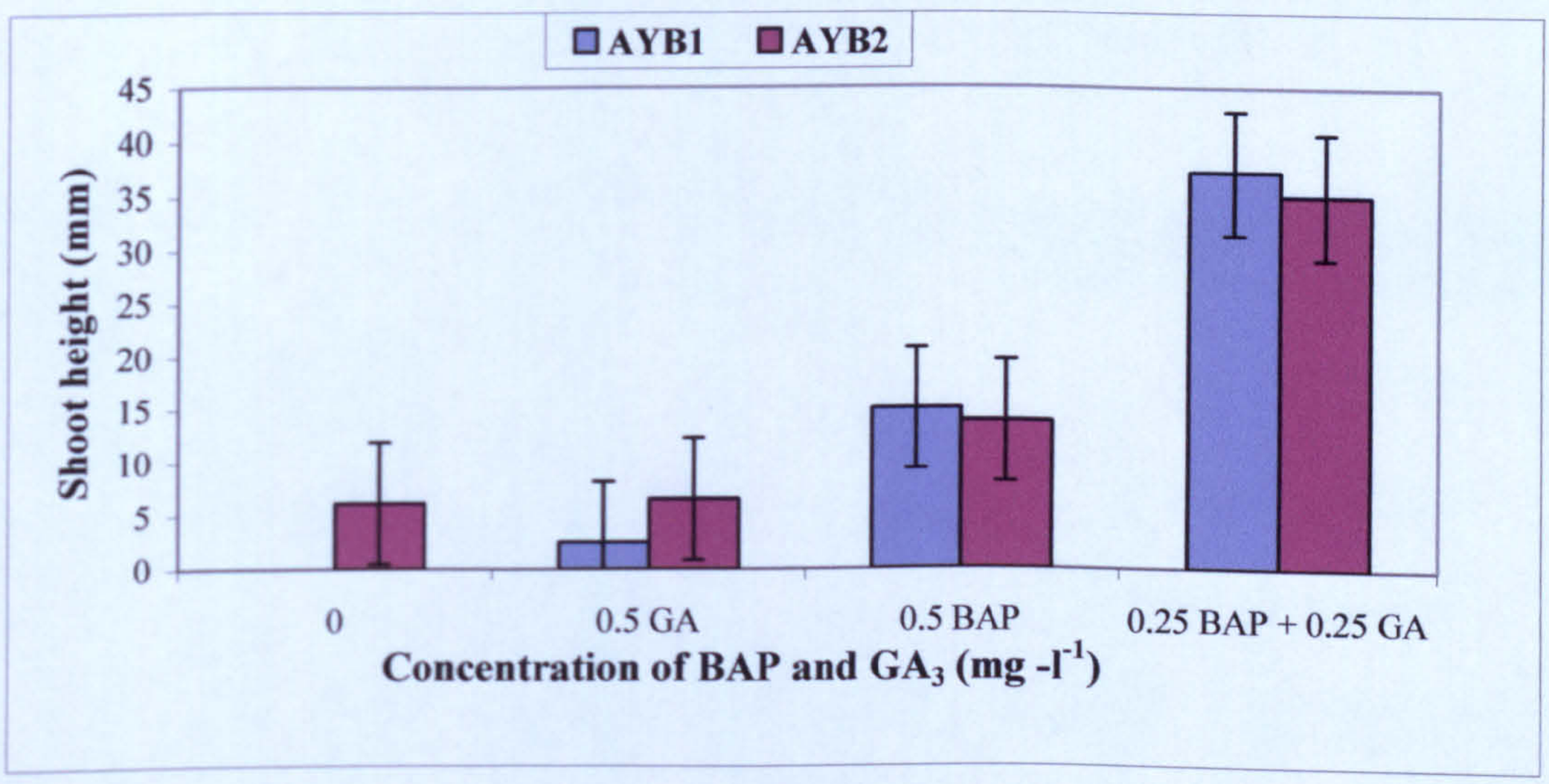

Figure 6.17 Effect of GA_3 on mean shoot height of AYB1 and AYB2 landraces cultured from axenic explants. Error bars = 2 *s.e.d.* (n=8) (Experiment 1)

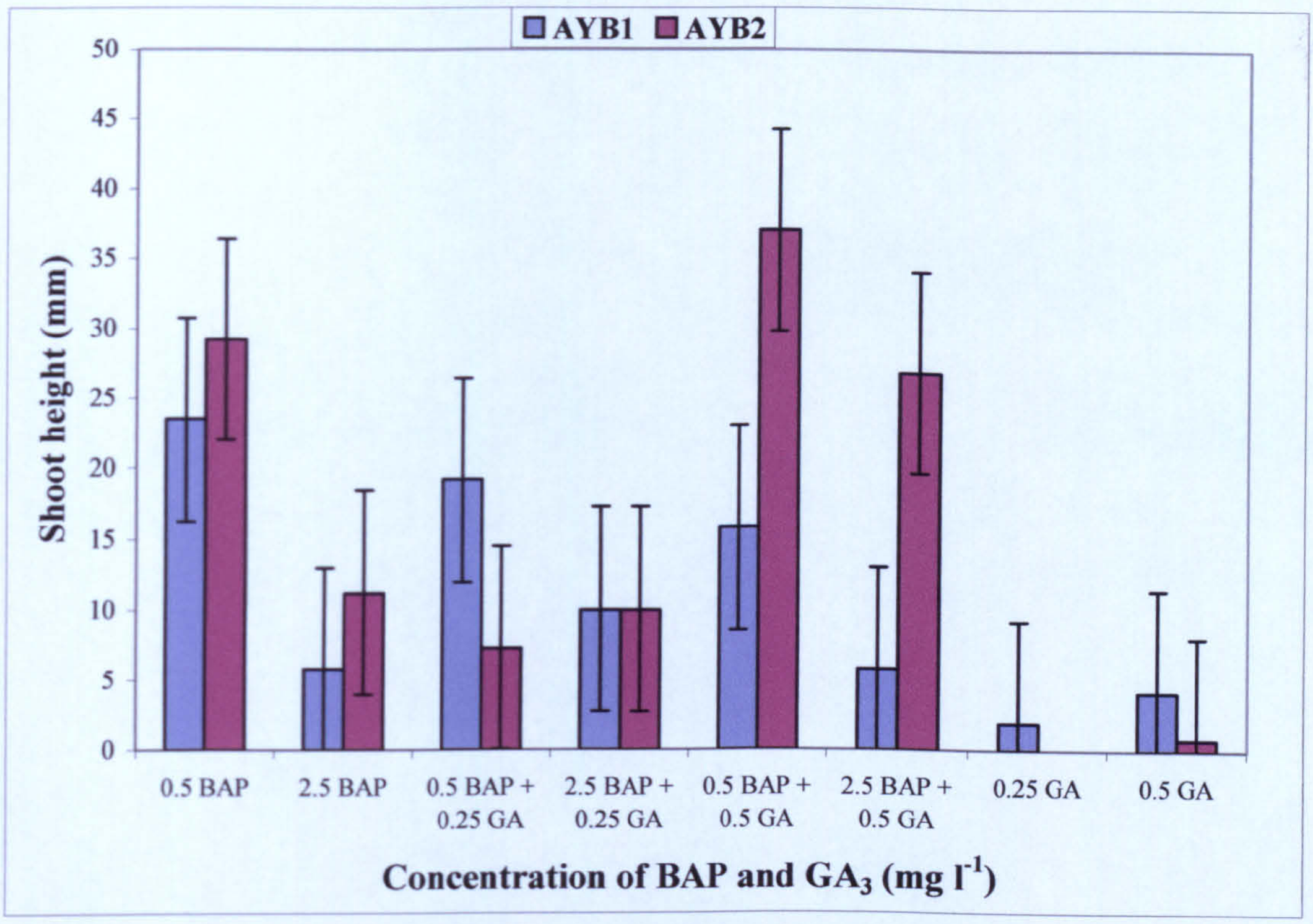

Figure 6.18 Effect of GA_3 on mean shoot height of AYB1 and AYB2 landraces cultured from axenic explants. Error bars = *s.e.d.* (n=7) (Experiment 2)

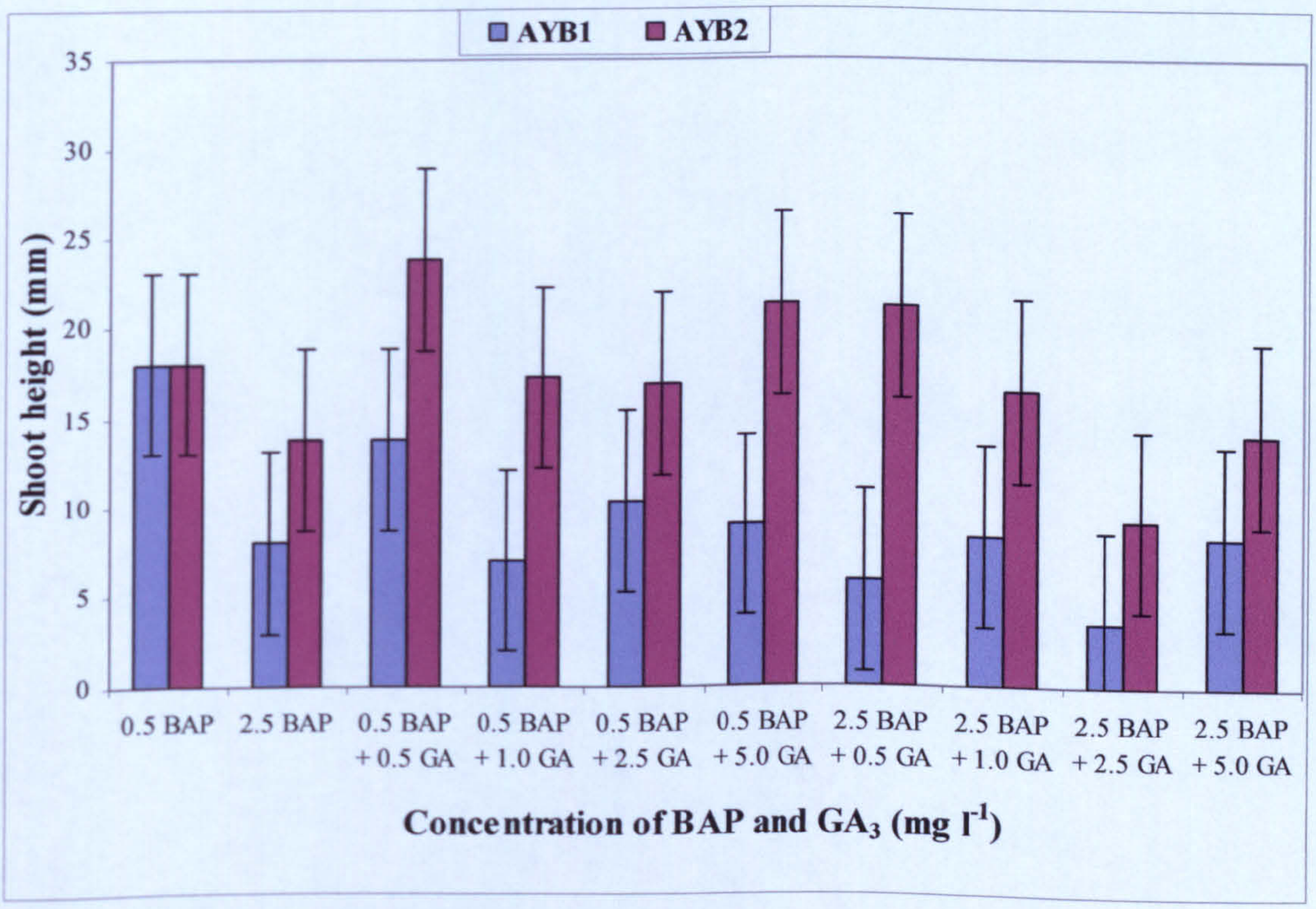

Figure 6.19 Effect of GA_3 on mean shoot height of AYB1 and AYB2 landraces cultured from axenic explants. Error bars = 2 *s.e.d.* (n=8) (Experiment 3)

Compared with the media containing BAP alone, there was no reduction in callus proliferation on media containing BAP + GA_3 (Tables 6.15, 6.16 and 6.17; Figures 6.20, 6.21 and 6.22). In contrast, growth of callus was very low in media without PGRs or with GA_3 alone. In terms of callus weight, a combination of PGRs in Experiments 1 and 2 did not produce a significant reduction, but in Experiment 3 there was some trend towards low callus production ($F_{(9,139)} = 3.57$, $p<0.001$) as the concentration of BAP and GA_3 was increased in the media (Table 6.17). In Experiments 1 and 2, there was no difference between the landraces but, in Experiment 3, AYB2 produced heavier callus ($F_{(9,139)} = 4.56$, $p<0.001$) (Table 6.17).

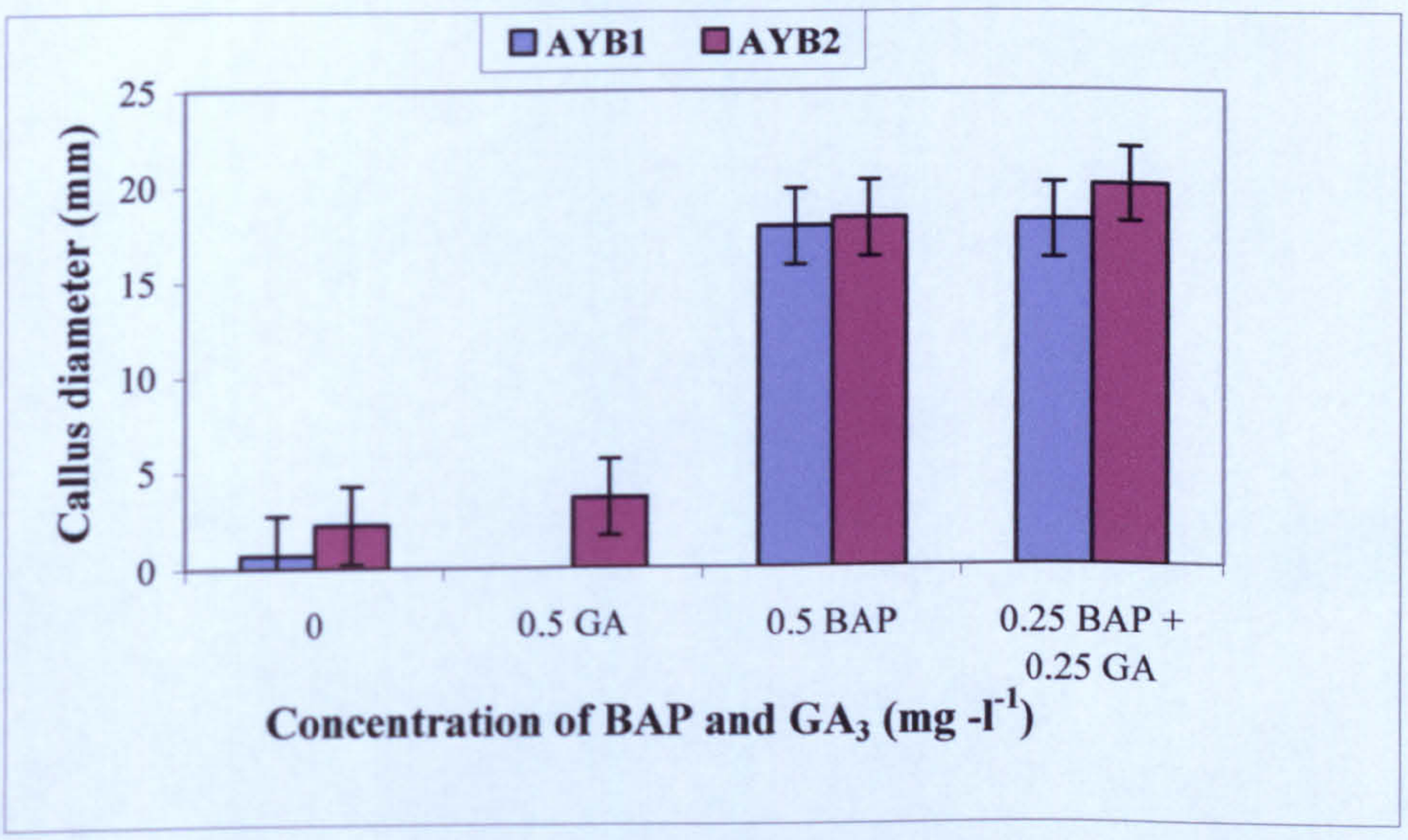

Figure 6.20 Effect of GA_3 on mean callus size (diameter) produced by AYB1 and AYB2 landraces cultured from axenic explants. Error bars = 2 *s.e.d.* (n=8) (Experiment 1)

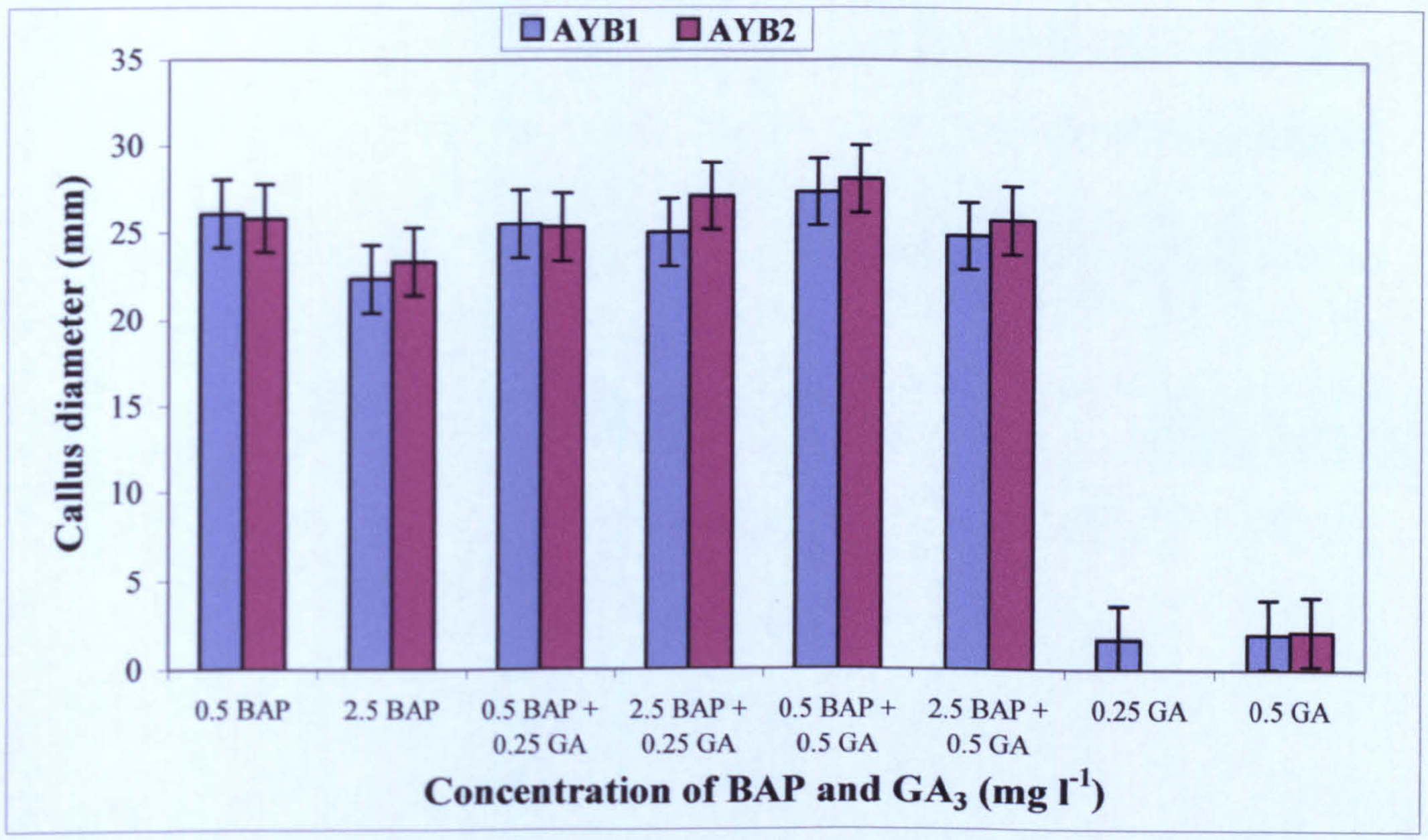

Figure 6.21 Effect of GA_3 on mean callus size (diameter) produced by AYB1 and AYB2 landraces cultured from axenic explants. Error bars = 2 *s.e.d.* (n=7) (Experiment 2)

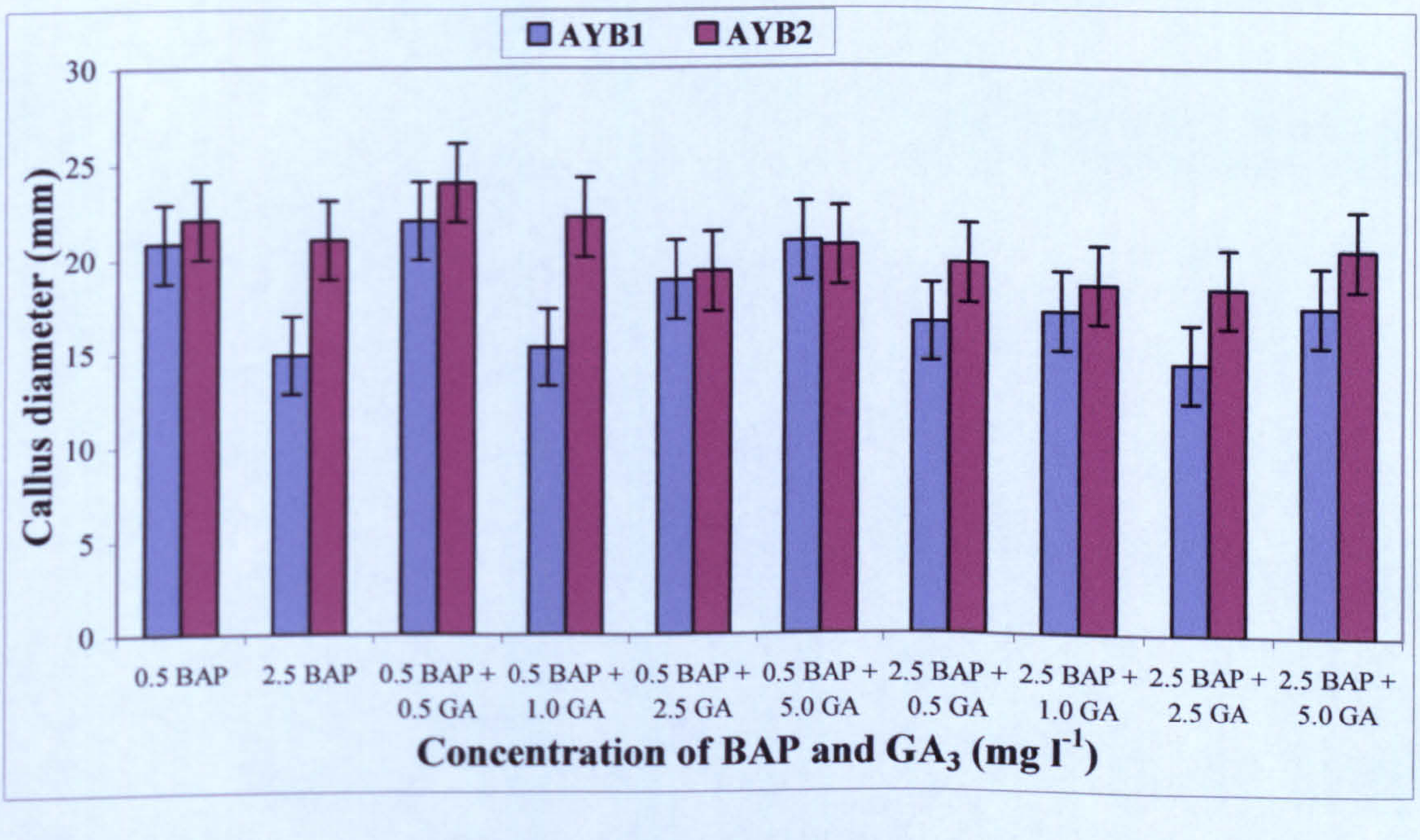

Figure 6.22 Effect of GA_3 on mean callus size (diameter) produced by AYB1 and AYB2 landraces cultured from axenic explants. Error bars = 2 *s.e.d.* (n=8) (Experiment 3)

6.3.4.3 Discussion

The synergistic effect of BAP and GA_3 on *in vitro* shoot growth observed in Experiment 1 confirmed previous findings (Pattnaik and Chand, 1997; Purohit and

Singhvi, 1998; Chitra and Padmaja, 1999; Vengadesan *et al.*, 2002), however, a reduction in callus proliferation was not observed. The concentrations of BAP and GA_3 were increased in the subsequent experiments to try to encourage shoot elongation and reduce callus proliferation, but the benefit was not clear suggesting that the concentrations may be critical. Chitra and Padamaja (1999) observed the elongation of *in vitro* grown shoots and sprouting of axillary buds of mulberry (*Morus indica* L.) when GA_3 (0.05 mg l^{-1}) was added to medium with elevated BAP concentration (4.0 mg l^{-1}). Without the addition of GA_3, the shoots did not elongate and this was also observed in the present study.

6.4 Rooting of *in vitro* shoots and acclimation of plantlets

Rooting of *in vitro*-derived plants is an important step in micropropagation as the ultimate goal is to have the plants grow *in vivo*. Although some species easily form adventitious roots on shoots produced *in vitro*, other species may require specific treatments before they can grow roots hence the third stage of micropropagation (George and Sherrington, 1984).

6.4.1 Effect of auxins and auxin concentration on adventitious root formation *in vitro*

Auxins are the most commonly applied PGRs to stimulate rooting and their potency varies with type of auxin, concentration and plant genotype (Pierik, 1987). Nikam and Shitole (1997) achieved root formation on shoots of niger (*Guizotia abyssinica*) grown on hormone-free media as well as media with hormones and found no significant difference in the numbers of rooted shoots on media with varying concentrations of IAA and NAA. However, the best root growth in all shoots was in the presence of 0.5 mg l^{-1}. Girija *et al.* (1999) recorded the best rooting with IBA at all concentrations tested (0.5-2.5 mg l^{-1}), compared to IAA and NAA which promoted callus at the basal cut end of firecracker plant/flower (*Crossandra infundibuliformis*). Geetha *et al.* (1997b) demonstrated auxin requirement in rooting of black gram (*Vigna mungo*) when shoots that failed to root on media devoid of auxin for 15 d rooted within 15-20 d of culture on medium containing different concentrations of IBA (0.1-5.0 mg l^{-1}). They observed maximum percentage rooting was with 3.0 mg l^{-1} IBA.

Rooting of axenic shoots of AYB1, AYB2, AYB3, AYB4 and AYB5 established in experiments reported in Section 6.2.1 was tested in three experiments. Experiment 1 tested the hypothesis that rooting of shoots is controlled by auxin application method whereas Experiments 2 and 3 tested the hypotheses that auxins are required to induce rooting of shoots *in vitro*, different auxins vary in their ability to induce rooting and rooting of shoots depends on auxin concentration.

6.4.1.1 Materials and methods

In Experiment 1, cultured shoots of AYB1, AYB2, AYB3 and AYB4 were transferred to half strength MS medium containing no auxin or IBA at 1.0 mg l^{-1}. The following treatments were used, namely (i) continuous culture of shoots on medium without auxin (no pulse treatment), (ii) culture of shoots on medium containing auxin for one day then transferred to medium containing no auxin (1d pulse treatment) and (iii) culture of shoots on medium containing auxin for 7d before transfer to medium without auxin. In experiment 2, shoots of AYB1, AYB2, AYB3 and AYB4 were rooted on half MS containing IBA and NAA at 1.0, 1.5 and 2.0 mg l^{-1}. In Experiment 3, shoots of AYB1, AYB2, AYB3, AYB4 and AYB5 were rooted on half MS containing IBA, NAA and 2.4-D at 3.5 mg l^{-1}.

The experiments were laid out as complete randomised designs with five replicates (culture vessels each containing one shoot) per treatment. The cultures were incubated for 6 weeks under conditions described in Chapter 3, Section 3.7.1 after which the number of shoots producing roots were recorded and rooted shoots transferred to equal volume peat and Perlite compost mix (Chapter 3, Section 3.5.2) in 8 cm plastic pots (Richard Sankey and Son, Nottingham, UK) and kept in a high humidity environment provided by polythene propagation boxes described in Chapter 3, Section 3.5.2.

6.4.1.2 Results

In the first experiment, rooting of AYB shoots was very low with no rooting observed in the control cultures that did not receive auxin and up to 7 out of 20 shoots rooting with auxin treatments (Table 6.18). In the second experiment, IBA appeared to be better for root induction than NAA (Table 6.19). When IBA, NAA

and 2,4-D were tested at the higher concentration of 3.5 mg l^{-1}, differences between IBA and NAA were not apparent, however, no rooting occurred in the presence of 2,4-D (Table 6.20). Differences in rooting between the landraces were not consistent between the three experiments.

Table 6.18 Number of shoots of AYB1, AYB2, AYB3 and AYB4 landraces rooting on half strength MS without IBA pulse (control) and with 1 and 7 d pulses on half strength MS plus 1 mg l^{-1} IBA. (n=5)

Genotype	No pulse	1 day pulse	7 day pulse	Total
AYB1	0	2	1	3
AYB2	0	0	1	1
AYB3	0	0	3	3
AYB4	0	0	2	2
Total	0	2	7	

Table 6.19 Effect of auxin type and concentration on number of shoots of AYB1, AYB2, AYB3 and AYB4 landraces forming roots. (n=5)

Landrace	IBA (mg l^{-1})			NAA (mg l^{-1})		
	1.0	1.5	2.0	1.0	1.5	2.0
AYB1	1	2	1	1	1	3
AYB2	2	3	2	1	1	2
AYB3	2	2	3	2	1	2
AYB4	0	2	3	1	2	0
Total	5	9	9	5	5	7

Table 6.20 Effect of auxin type at 3.5 mg l^{-1} on number of shoots of AYB1, AYB2, AYB3, AYB4 and AYB5 landraces forming roots. (n=5)

Landrace	Auxin			Total
	IBA	2.4D	NAA	
AYB1	2	0	1	3
AYB2	5	0	4	9
AYB3	0	0	1	1
AYB4	1	0	0	1
AYB5	0	0	1	1
Total	8	0	7	

When rooted plants were transferred to compost, vigorous plantlets with well developed roots and shoots (Plate 6.4B) compared to smaller shoots (Plate 6.4A)

grew successfully into normal plants and were subsequently used as clonal stock plants (Plate 6.4C).

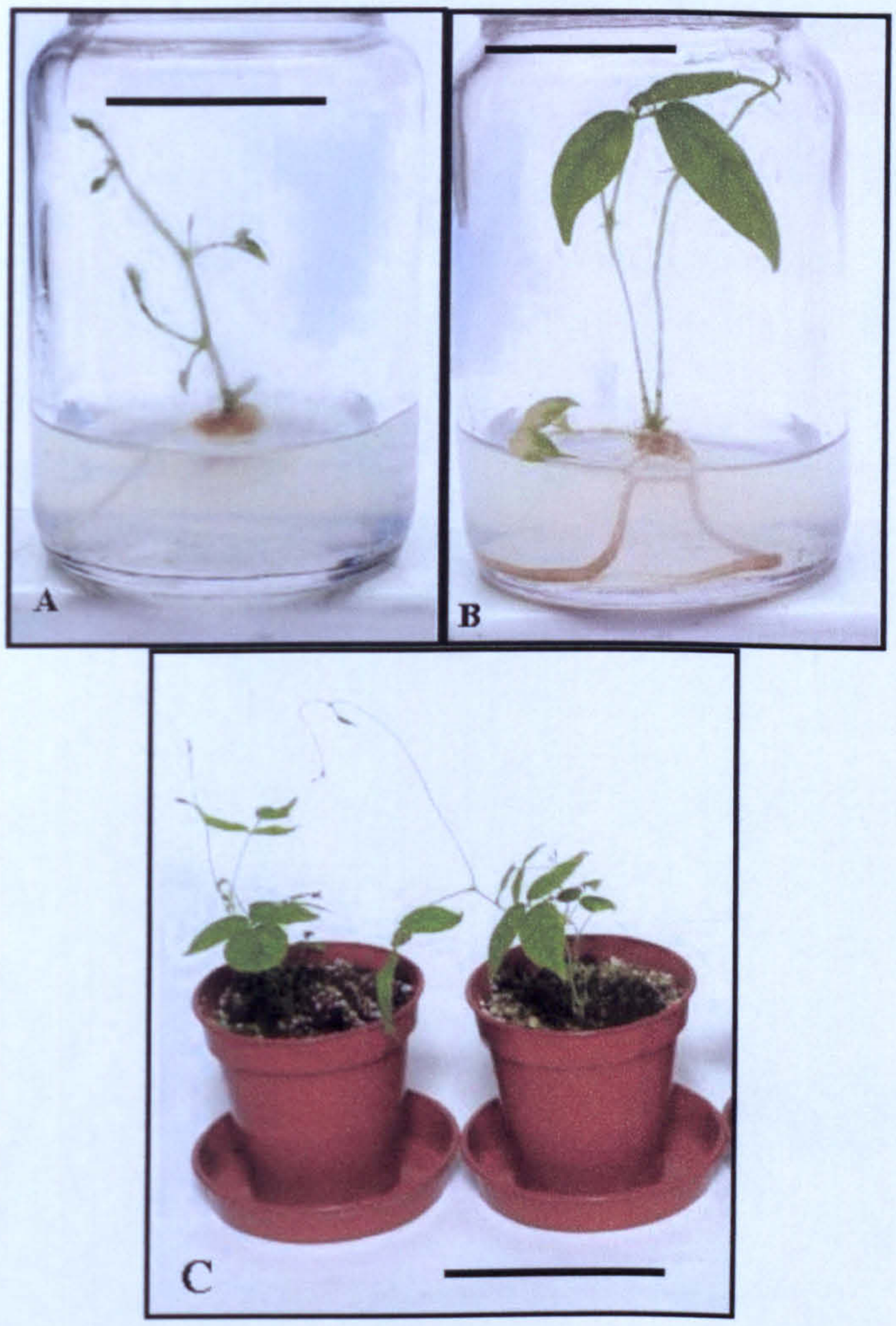

Plate 6.4 Shoots rooted *in vitro* and growing in compost after successful acclimation. Bars = 25 mm (A, B), 9 cm (C).

6.4.1.3 Discussion

The absence of rooting in the control treatment indicated the need for auxin for the rooting of AYB. Some plants are known to have a high endogenous production of auxin and thus do not require any exogenous application, while others have a requirement for exogenous application (Pierik, 1987). It is likely that, based on the observations to date, AYB may conform to the latter category of plants. This is perhaps unexpected when it is considered that the large amounts of callus produced by AYB explants may reflect high levels of endogenous auxins. IBA appeared to be relatively better for root induction than NAA and 2,4-D, as has been reported for most plants (Geetha *et al*., 1997b; Girija *et al*., 1999).

6.4.2 Effect of PP333 on rooting of shoots of AYB landraces

Gibberellins are naturally occurring growth substances that induce growth but are not generally known to favour organ initiation (Rout *et al.*, 2000) and usually inhibit adventitious root formation (George and Sherrington, 1984; Pierik, 1987). Rooting of species previously known to be slow to root has been achieved with shoots exposed to gibberellin biosynthesis inhibitors. Rooting was achieved on rhizome buds of *Lapageria rosea* that were proliferated in the presence of the gibberellin biosynthesis inhibitor paclobutrazol at 5μM (McKinless and Alderson, 1993). As a growth retardant, paclobutrazol may, however, also have deleterious effects on plant growth as observed in delayed seedling emergence and retarded vegetative growth of maize (*Zea mays*) (Khalil and Rahman, 1995) and in inhibition of rooting in bean (*Phaseolus vulgaris*) (Tari and Nagy, 1996).

Two experiments were carried out to test the hypothesis that PP333 promotes rooting by blocking the synthesis of gibberellins known to inhibit root formation. The application of PP333, a gibberellin biosynthesis inhibitor, at the shoot initiation and rooting stages was evaluated on rooting of AYB1 and AYB2 landraces.

6.4.2.1 Materials and methods

In the first Experiment, nodal explants were surface sterilised according to procedure described in Chapter 3, Section 3.4.4 and cultured on full strength MS medium containing 0.5 mg l^{-1} BAP and PP333 at 0, 0.75 and 1.5 mg l^{-1} for 5 weeks under conditions described in Chapter 3, Section 3.7.1. Shoots were then transferred to half strength MS medium containing 3.0 mg l^{-1} IBA for rooting. In Experiment 2, nodal explants were also surfaced sterilised according to procedure described in Chapter 3, Section 3.4.4 and cultured first on MS medium containing 0.5 mg l^{-1} BAP under conditions described in Chapter 3, Section 3.7.1 for five weeks and then transferred to half strength MS containing 3.0 mg l^{-1} IBA and PP333 at 0, 1.5 and 3.0 mg l^{-1}. In both experiments, the cultures were incubated for six weeks in conditions described in Chapter 3, Section 3.7.1 and evaluated every two days for root formation. Rooted plantlets were transferred to compost mix (described in Chapter 3, Section 3.5.2) in 8cm plastic pots and kept in a high humidity environment provided by polythene propagation boxes described in Chapter 3, Section 3.5.2.

6.4.2.2 Results

No benefit in rooting of shoots was derived from PP333 applied at either the shoot culture or the rooting stages (Table 6.21) and there was a marked retardation in the growth of shoots when PP333 was applied at the shoot culture growth stage (Plate 6.5A) and tissue deformation when applied at the rooting stage (Plate 6.6). Shoots rooted in the presence of PP333 produced more root primodia and less root extension (Plate 6.6).

Table 6.21 Effect of PP333 on *in vitro* rooting of shoots of AYB1 and AYB2.

Treatment	AYB1		AYB2	
	Number rooting (n=15)	**Percentage rooting**	**Number rooting (n=15)**	**Percentage rooting**
PP333 applied at shoot growth (mg l^{-1})				
0	3	20.0	10	66.0
0.75	3	20.0	8	53.3
1.5	2	13.3	6	40.0
PP333 applied at rooting (mg l^{-1})				
0	3	20.0	5	33.3
1.5	3	20.0	4	26.7
3.0	0	0	0	0

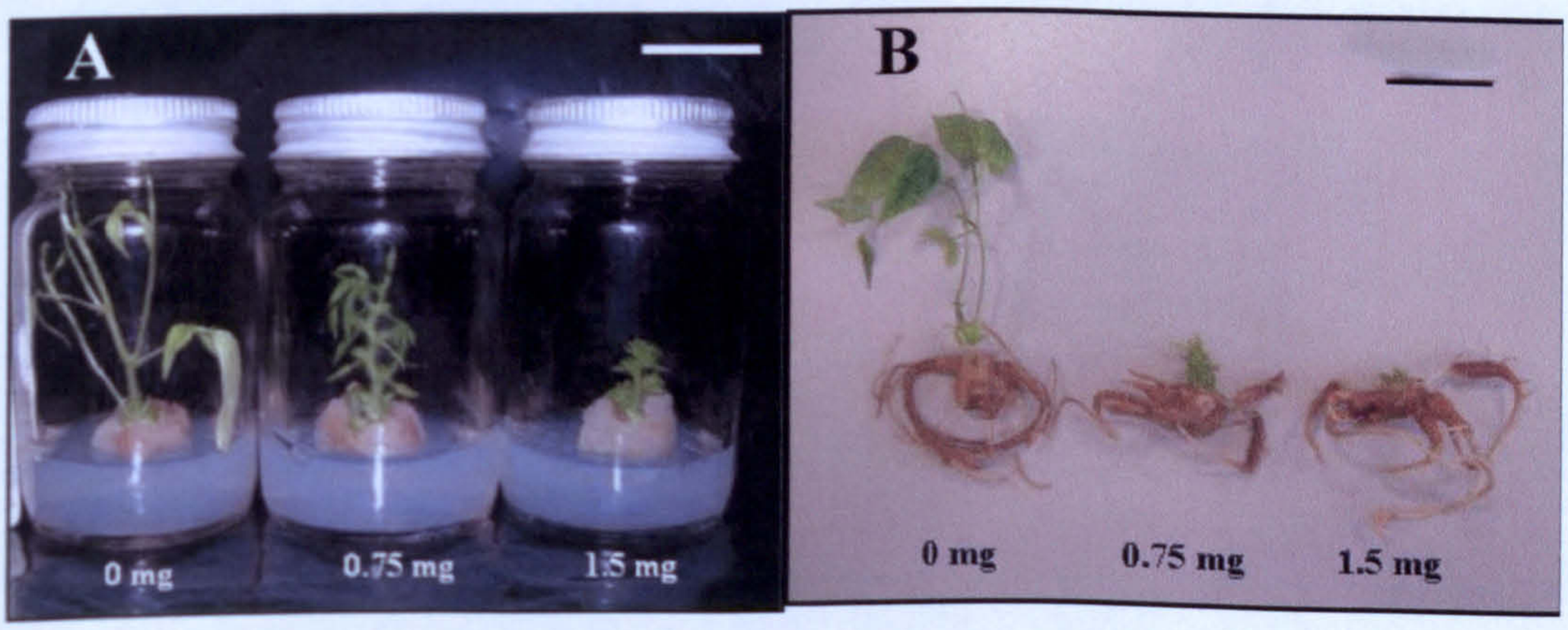

Plate 6.5 Effect of PP333 applied at shoot growth stage (A, shoots 5 weeks in initiation culture and B, shoots after rooting). Bars: A = 20 mm and B 30 mm.

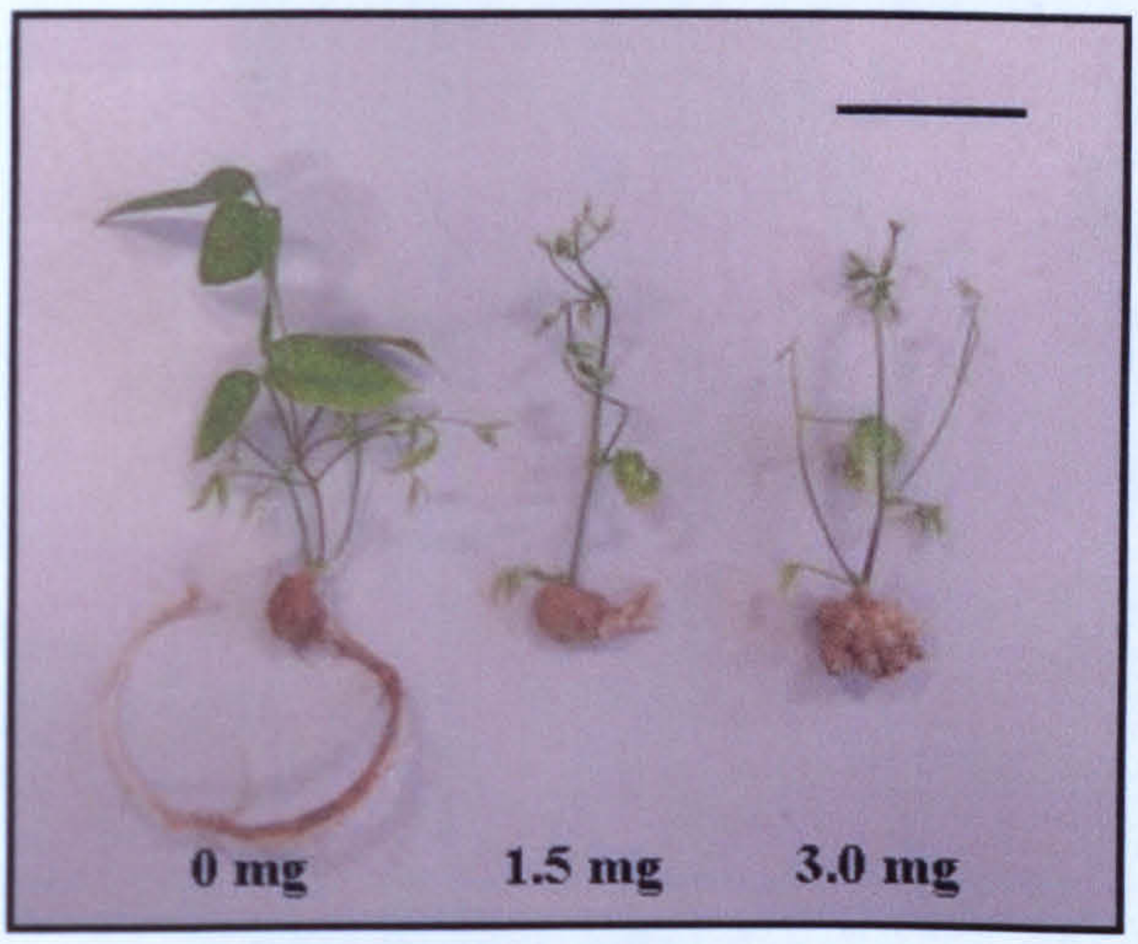

Plate 6.6 Effect of PP333 applied at rooting stage. Bar = 30 mm.

There was a negative effect of PP333 on acclimation as all shoots rooted in the presence of PP333 failed to develop into normal plants (Table 6.22). In contrast, shoots rooted in the absence of PP333 grew well after transfer to compost.

Table 6.22 Acclimation of plants of AYB1 and AYB2 landraces rooted *in vitro* after PP333 treatment.

Treatment	AYB1		AYB2	
	Potted plants	Surviving plants	Potted plants	Surviving plants
PP333 applied at shoot growth (mg l^{-1})				
0	2	2	9	4
0.75	5	0	6	0
1.5	0	0	4	0
PP333 applied at rooting (mg l^{-1})				
0	2	1	5	3
1.5	0	0	2	0
3.0	0	0	2	0

6.4.2.3 Discussion

Addition of PP333 to the culture medium did not improve the rooting of AYB shoots. Shoots that received PP333 either in the shoot growth medium or rooting medium showed signs of growth retardation and those that rooted did not survive transfer to compost. The size of a shoot seemed to play a major role in the establishment of rooted plants with less vigorous plants failing to grow *ex vitro*.

While many studies have reported beneficial effects of growth retardants such as PP333 on rooting, Khalil and Rahman (1995) observed delayed seedling emergence and retarded vegetative growth of maize (*Zea mays* L.) and Tari and Nagy (1996) recorded inhibited root formation in bean (*Phaseolus vulgaris* L.). Results with AYB are consistent with this negative action. While the negative effect of PP333 on AYB shoots could be due to its action as a growth retardant, it is also possible that its capacity to increase the concentration of endogenous IAA (Sebanek *et al.*, 1991) resulted in the combined effect of exogenous and endogenous auxin leading to toxicity.

CHAPTER 7
NODULATION AND NITROGEN FIXATION

7.1 Introduction

It is paradoxical that nitrogen (N) constitutes about 80% of the earth's atmosphere yet it is the most limiting nutrient for plant growth and the most deficient in soils. In an endeavour to redress the problem, mineral fertilizers are usually incorporated into soils although for various reasons it is generally difficult in most impoverished parts of the world such as Africa (Giller, 2001). However, in the developed world where fertilizer is less costly, there is fear of environmental contamination and loss of biodiversity due to increased fixation and mobilization of N through fertilizers and combustion (Frink *et al.*, 1999). Therefore, an essential element of agricultural sustainability is the effective management of N in the environment, which usually involves some use of biologically fixed N because N from this source is used directly by the host plant and is less susceptible to volatilisation, denitrification and leaching (Graham and Vance, 2000). One of the important contributions of AYB in mixed cropping systems is its contribution to soil productivity. The use of AYB and other legumes as cover crops has increased the efficiency of fertilizer utilization and contributed organic matter for maintenance of high soil productivity (Obiagwu, 1995b).

Legumes are known for their ability to fix atmospheric nitrogen through root nodules developed in association with some strains of bacteria of the *Rhizobiaceae*. The fast growing *Rhizobium* and the slow growing *Bradyrhizobium* are the two types of bacteria species involved in nodulation of legumes. Some strains of rhizobia may be termed compatible if they form a symbiosis with some other legume species or incompatible if they cannot form a symbiosis with the host plant in question. Specificity of the microsymbiont is very important, as it has been shown that if such a strain is present in sufficient numbers, certain legumes such as soybean can obtain their total nitrogen needs from the air (Panzieri *et al.*, 2000). Plant species vary in their degree of specificity in the requirement of effective rhizobial strains, and hence it is important to evaluate and select the most effective strain to use as an inoculant

for AYB. Deliberate selection and introduction of adapted rhizobial genotypes can also be used as a tool to optimise the legume symbiosis under stressful situations (Howieson and Ballard, 2004). Therefore, an ideal legume crop may not only be a high yielding one, but also one capable of symbiotic relations with most nitrogen fixing bacteria.

Nitrogen fixation (% nitrogen derived from atmosphere when only above ground parts are considered) varies amongst grain legumes grown under different conditions, but is quite substantial with ranges of 16-92% (*Arachis hypogea*), 0-96% (*Cicer arietinum*), 12-100% (*Glycine max*), 85-91% (*Lathyrus sativus*), 9-91% (*Lens culinaris*), 0-88% (*Cajanus cajan),* 0-73% *(Phaseolus vulgaris)*, 0-100% *(Vigna radiata*) and 32-76% (*Vigna unguiculata*) (Giller, 2001). While the legume–rhizobial symbiosis is understood for many major legume crop species, it has been less studied for non-conventional and under-utilised crops such as AYB, because they attract less research interest. An endosymbiont, characterized and designated as *Bradyrhizobium* sp. AUEB20, isolated from the Ethiopian tree *Erythrina brucei* has been reported to form a small number of large branched (each about 1 cm in diameter) indeterminate N_2-fixing (1.02 µmoles g^{-1} h^{-1} C_2H_4) nodules with AYB (Assefa and Kleiner, 1997). This strain was also found to be promiscuous with respect to other tropical legumes since it also infected and caused nitrogen fixation on *Cajanus cajan* (21.4 µmoles g^{-1} h^{-1} C_2H_4), *Dolichos lablab* (8.9 µmoles g^{-1} h^{-1} C_2H_4), *Vigna unguiculata* (3.6 µmoles g^{-1} h^{-1} C_2H_4) and *Vigna subterranean* (1.6 µmoles g^{-1} h^{-1} C_2H_4). The AYB nitrogen fixation in association with strain AUEB20 was less efficient compared to the other legumes tested, hence the need to evaluate more rhizobial strains to identify efficient ones.

The purpose of this study was to determine the response of AYB to inoculation with nitrogen fixing bacteria and to identify the host/microsymbiont combination for optimal performance of the N-fixing symbiosis. The hypothesis guiding the study was that, various rhizobial strains known to nodulate tropical legumes will cause formation of nitrogen fixing nodules on AYB landraces to sustain plant growth without need for nitrogen supplementation. The objectives were as follows:

- Determination of the infectivity (nodulation ability/formation of nodules) of various rhizobial strains on AYB landraces
- Establish the effectivity (nitrogen fixation capacity) of nodulation by nodulating strains of rhizobia
- Quantify contribution of the various strains to AYB growth and yield.

Three experiments were carried out as described below.

7.2 Nodulation and nitrogen fixation of AYB with *Bradyrhizobium* sp. AUEB20 (Experiment 1)

This experiment was conducted as a preliminary trial to establish the infectivity and effectivity of strain AUEB20 on 2 AYB landraces, since it is recognised that plant species or genotypes vary in their degree of specificity with respect to effective rhizobial strains.

7.2.1 Materials and Methods

The experiment was conducted in the glasshouse (conditions described in Chapter 3, Section 3.7.3) during the summer of 2003 (May-August) with AYB1 and AYB2 landraces. There were 2 treatments in this experiment, specifically; control treatment (uninoculated plants) and treated (inoculated plants). Inoculum preparation and plant inoculation were according to the procedure described in Chapter 3, Section 3.6.3. The inoculum used had a bacterial concentration $>3.0 \times 10^{11}$ cells ml^{-1}. Plants were grown in 13 cm OS plastic (thermoformed polypropylene) pots (Soparco, Condé sur Huisne, France) placed in plastic trays (described in Chapter 3, Section 3.6.3) to trap inoculum-contaminated water. The experiment was set up with 20 plants inoculated and 10 uninoculated (control) plants for each landrace. Five plants were selected randomly every 28 d to check for nodule development. The experiment was terminated 12 weeks after inoculation and plants assessed for presence of nodules.

7.2.2 Results

Nodulation was not observed in any of the landraces inoculated with *Bradyrhizobium* strain AUEB20. Inoculated and uninoculated plants were stunted in their growth.

7.3 Evaluation of nodulation and nitrogen fixation of AYB landraces by different strains of rhizobia (Experiment 2)

This experiment was set up to evaluate compatibility of AYB landraces with other rhizobial strains since, in the preliminary trial (Experiment 1), strain AUEB20 did not nodulate AYB plants contrary to observations reported by Assefa and Kleiner (1997).

7.3.1 Materials and methods

The experiment was conducted in the controlled environment room (conditions described in Chapter 3, Section 3.7.2) during the winter of 2003/04 (December-February). The following rhizobial strains (described in Chapter 3, Section 3.6.1), AUEB20, ORS302, CP279, NGR234 and ANU240 were used with strength > 3.0×10^{11} cells ml^{-1}, including a control treatment of uninoculated plants. Plants were inoculated according to the procedure described in Chapter 3, Section 3.6.3 and grown in 9 cm OS plastic (thermoformed polypropylene) pots placed in plastic trays to trap inoculum-contaminated water. Thirty plants per strain were used and a control treatment with 10 uninoculated plants for each landrace was set up alongside inoculated plants. Measurements were taken after 12 weeks of incubation with a total of 15 plants per treatment randomly sampled to assess the percentage nodulation. Five plants from each treatment were used to record number of nodules, nodule mass, nitrogen fixation capacity (nitrogenase activity and tissue nitrogen content) and leaf chlorophyll content for each landrace. Data was subjected to analysis of variance (ANOVA) using Genstat (Chapter 3, Section 3.9) at a 5% level of significance.

7.3.2 Results

Formation of nitrogen fixing nodules on the 2 landraces used was recorded with strains ORS302, CP279 and NGR234 and the results are presented below. Strains AUEB20 and ANU240 did not cause nodulation of host plants and hence were not included in data analysis.

7.3.2.1 Nodule formation on AYB landraces

Two rhizobial strains, ORS302 and CP279, successfully infected roots of both AYB landraces by inducing nodulation in all 15 plants sampled, while strain NGR234 caused nodulation on only 6 and 7 plants of AYB1 and AYB2 respectively (Table 7.1; Plate 7.1). Amongst the strains that caused nodule formation, the number of nodules per plant differed significantly ($F_{(2,24)} = 27.01$, $p<0.001$) with strain ORS302 inducing the largest number [349 (AYB1) and 365 (AYB2)], followed by CP279 [97 (AYB1) and 128 (AYB2)] and NGR234 the least [5 (AYB1) and 12 (AYB2)].

The strains differed significantly ($F_{(2,24)} = 44.49$, $p<0.001$) in dry weight of nodules and the trend was similar to that observed with number of nodules where strains ORS302 performed better, followed by strain CP279. Strains ORS302 caused formation of greater nodule dry weight of 0.211 and 0.32 g plant^{-1} on AYB1 and AYB2 respectively, in relation to strain CP279 of 0.094 g plant^{-1} (AYB1) and 0.123 g plant^{-1} (AYB2). However, there was also some significant ($F_{(1,24)} = 5.58$, $p<0.05$) difference in nodule dry weight between landraces. AYB2 was the most responsive landrace, since it generally performed better than AYB1 when inoculated with the 2 most efficient rhizobial strains (ORS302 and CP279).

Table 7.1 Nodulation (number nodulated plants, mean number of nodules per plant and mean nodule dry weight per plant) of AYB landraces by different rhizobial strains

Treatment (rhizobial strain)	Number of nodulated plants (n=15)		Number of nodules per nodulated plant (n=5)		Nodule dry weight (g) per plant (n=5)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Uninoculated	0	0	-	-	-	-
ORS302	15	15	349	365	0.211	0.320
CP279	15	15	97	128	0.094	0.123
NGR234	6	7	5	12	0.046	0.046
s.e.d.	-		68.9		0.034	
d.f.	-		24		24	
Significance (5%)						
Landrace	-		0.654		0.027	
Rhizobial strain	-		<0.001		<0.001	
Interaction	-		0.970		0.080	

Plate 7.1 AYB2 plant showing root nodules following inoculation with rhizobial strain ORS302. Bars, main picture = 100 mm; insert = 20 mm.

7.3.2.2 Nitrogen fixation capacity of AYB landraces

All the plants that produced nodules were active in nitrogen fixation, but fixation capacity differed between rhizobial strains and between landraces (Table 7.2). On a nodule dry weight basis, nitrogen fixation ability of nodules revealed by nitrogenase activity differed significantly ($F_{(2,24)}$ = 6.89, p<0.01) between strains. Strain NGR234, which induced the least number of nodules on host plants, was more active in nitrogen fixation on AYB2 than strains ORS302 and CP279 and was also more efficient in AYB1 than strain CP279. Nitrogenase activity of 7.04 µmoles C_2H_4 g^{-1} h^{-1} on AYB2 nodules caused by strain NGR234 was the greatest, while nodules induced by strain CP279 were the least active with 0.46 µmoles C_2H_4 g^{-1} h^{-1} for both AYB1 and AYB2. However, when nitrogen fixation was assessed on a per plant basis, the difference between rhizobial strains was highly significant ($F_{(2,24)}$ = 29.20, p<0.001), but strain ORS302 that evoked more nodules per plant performed better than the strains CP279 and NGR234 which caused the formation of less nodules. Genotypic variation on nitrogen fixation was evident as the landraces also differed significantly ($F_{(1,24)}$ = 23.63, p<0.001) from each other, where landrace AYB2 with more nodules per plant had a high nitrogenase activity compared to AYB1 which had smaller number of nodules per plant. The interaction between rhizobial strain and landrace was also highly significant ($F_{(2,24)}$ = 10.41, p<0.001).

Accumulation of nitrogen in host plant tissues also revealed a significant ($F_{3,32)}$ = 44.44, p<0.001) difference between rhizobial strains as well as significant interaction ($F_{(3,32)}$ = 3.95, p<0.05) between strain and landrace (Table 7.2). Plants inoculated with strain CP279 yielded more nitrogen (AYB1, 3.01 % and AYB2, 2.62 %) than strains ORS302 (AYB1, 1.93 % and AYB2, 2.40 %) and NGR234 (AYB1, 1.72 % and AYB2, 1.93 %). Difference in rhizobial strain/host plant association was evident, as AYB1 accumulated more nitrogen with strain CP279, while AYB2 yielded more nitrogen when inoculated with strains ORS302 and NGR234. As might be expected, uninoculated plants (control) had the lowest nitrogen content (Table 7.2).

Table 7.2 Mean nitrogen fixation of AYB landraces nodulated by different rhizobial strains estimated by the Acetylene Reduction Assay (ARA) and tissue nitrogen content (%). (n=5)

Treatment (rhizobial strain)	ARA (µmoles C_2H_4 per nodule dry wt (g) h^{-1})		ARA (µmoles C_2H_4 per plant h^{-1})		Tissue nitrogen content (%)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Uninoculated	-	-	-	-	1.28	1.46
ORS302	2.15	4.63	0.37	1.39	1.93	2.40
CP279	0.46	1.99	0.04	0.13	3.01	2.62
NGR234	1.98	7.04	0.10	0.32	1.72	1.93
s.e.d.	1.272		0.158		0.182	
d.f.	24		24		32	
Significance (5%)						
Landrace	<0.001		<0.001		0.190	
Rhizobial strain	0.004		<0.001		<0.001	
Interaction	0.151		<0.001		0.017	

7.3.2.3 Chlorophyll content

Total leaf chlorophyll content (mg g^{-1} fresh weight) differed significantly between strains ($F_{3,32)}$ = 119.61, p<0.001) and between landraces ($F_{1,32)}$ = 5.28, p<0.05) (Figure 7.1). Surprisingly, strain NGR234 yielded more chlorophyll than the other strains in both landraces although it induced formation of the least number of nodules (Figure 7.1). Uninoculated plants were the lowest in leaf chlorophyll content as was the case with tissue nitrogen content. The interaction between landrace and strain was also significant ($F_{3,32)}$ = 119.61, p<0.01), where AYB1 performed as good as AYB2, with strain CP279 whereas AYB2 had higher chlorophyll content with strain NGR234 and ORS302 (Figure 7.1).

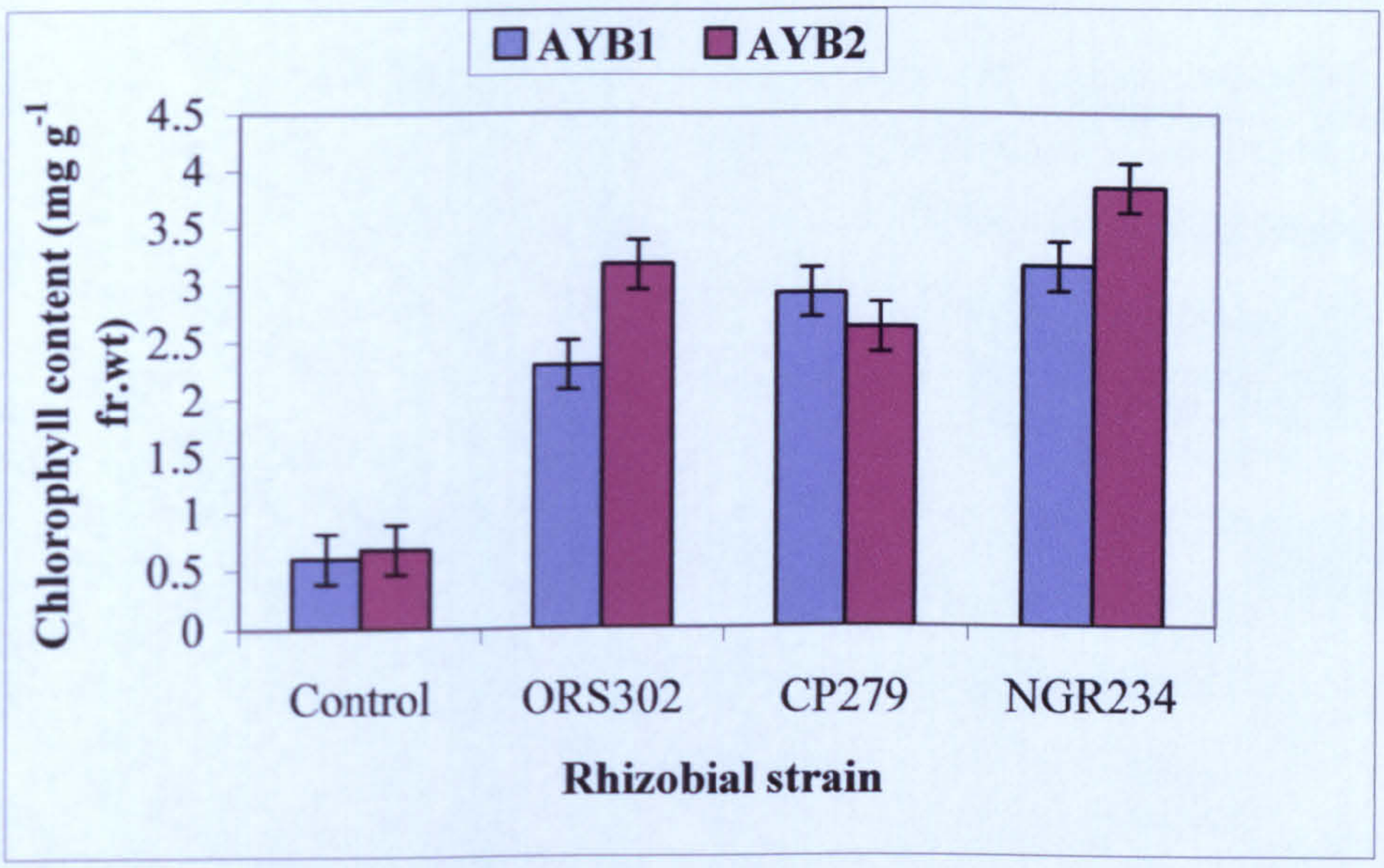

Figure 7.1 Effect of different rhizobia strains on total leaf chlorophyll of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=5)

7.4 Evaluation of symbiotic effectiveness of different rhizobial strains nodulating AYB landraces (Experiment 3)

In the second experiment, 3 strains were found to form a symbiosis with AYB landraces. The purpose of Experiment 3 was to evaluate the contribution or effectiveness of that symbiosis in nitrogen fixation and growth of AYB landraces.

7.4.1 Materials and methods

The experiment was conducted in the glasshouse (conditions described in Chapter 3, Section 3.7.2) in the summer of 2004 (June-August) with AYB1 and AYB2 landraces using 3 rhizobial strains (ORS302, CP279 and NGR234) found to form nitrogen fixing nodules in Experiment 2. Inocula preparation and plant inoculation were carried out according to the procedure described in Chapter 3, Section 3.6.3. Twenty plants per strain were used and a control treatment with 10 uninoculated plants for each landrace was set up alongside inoculated plants. Plants were grown in 13 cm OS plastic (thermoformed polypropylene) pots placed in plastic trays to trap inoculum-contaminated water. Measurements were taken after 12 weeks of incubation. A total of 10 plants per treatment were sampled randomly to assess percentage nodulation, while for nodule formation (number of nodules and nodule mass), nitrogen fixation [tissue nitrogen content (%), total shoot nitrogen content and

fixed nitrogen per shoot] and plant growth parameters (dry matter accumulation and leaf area) six plants were used because of senescence of some of the control plants. Fixed nitrogen per shoot was calculated as the difference between total shoot nitrogen content of inoculated and uninoculated (control) plants [i.e. total shoot nitrogen content (mg) of inoculated plants less total shoot nitrogen content (mg) of uninoculated plants]. Shoot dry weight increase was estimated by expressing shoot dry weight of inoculated plants as a percentage of the shoot dry weight of uninoculated plants (control). Data was subjected to analysis of variance (ANOVA) using Genstat (Chapter 3, Section 3.9) at a 5% level of significance.

7.4.2 Results

A substantial contribution of different rhizobial strains to nitrogen fixation and growth of AYB landraces was observed in this experiment.

7.4.2.1 Nodule formation

Nodules were observed to have formed within 28 d of inoculation with strains ORS302 and CP279. At the termination of the experiment, strains ORS302 and CP279 had nodulated all the plants of the 2 landraces, while strain NGR234 caused nodule formation only on AYB2, but on a relatively smaller proportion (Table 7.3). There was a significant ($F_{(2,30)} = 76.95$, $p<0.001$) difference in number of nodules recorded between plants inoculated with different rhizobial strains but not between the landraces. Strain CP279 caused the greatest number (319 and 329) of nodules plant^{-1} on both landraces (AYB1 and AYB2 respectively), while strain ORS302 followed with 186 and 182 nodules for AYB1 and AYB2, respectively (Table 7.3). A trend similar to that displayed in number of nodules was observed in nodule dry weight per plant where the difference between rhizobial strains was significant ($F_{(2,30)} = 56.60$, $p<0.001$). Strain CP279 caused the formation of heavier nodules per plant (AYB1, 1.09 g and AYB2, 1.63 g) than the other two strains. NGR234 was the least effective strain in terms of both nodule number and nodule mass. Observations on nodule dry weight showed that, unlike the number of nodules, landraces differed significantly ($F_{(2,30)} = 9.88$, $p<0.01$) with AYB2 producing a greater nodule mass than AYB1 with all rhizobial strains.

Table 7.3 Nodulation [number of nodulated plants, mean number of nodules per plant and mean nodule dry weight (g) per plant] of AYB landraces by different rhizobial strains.

Treatment (rhizobial strain)	Number of nodulated plants (n=10)		Number of nodules per plant (n=6)		Nodule dry weight (g) per plant (n=6)	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Uninoculated	0	0	-	-	-	-
ORS302	10	10	186	182	0.94	1.08
CP279	10	10	319	329	1.09	1.63
NGR234	0	6	0	26	0	0.25
s.e.d.	-		35.6		0.169	
d.f.	-		30		30	
Significance (5%)						
Landrace	-		0.607		0.004	
Rhizobial strain	-		<0.001		<0.001	
Interaction	-		0.838		0.257	

7.4.2.2 Nitrogen fixation

As with other parameters, nodulation with ORS302 and CP279 improved remarkably the nitrogen nutrition of AYB landraces (Table 7.4). There was a significant ($F_{(3,40)}$ = 84.89, p<0.001) difference in tissue nitrogen accumulation between plants inoculated with strains ORS302 and CP279 and those inoculated with NGR234 and uninoculated ones (control). Plants inoculated with strains ORS302 and CP279 accumulated more nitrogen in AYB1 (3.00 and 3.02 % respectively) and AYB2 (3.18 and 3.05 % respectively), while relatively small amounts were recorded with strain NGR234 (AYB1; 1.38 % and AYB2; 2.13 %) and the control treatment (AYB1; 1.22 % and AYB2; 1.18 %) (Table 7.4). While strain NGR234 did not perform to the level of the other strains, it still differed significantly from the control treatment. Total shoot nitrogen content followed a similar trend as tissue nitrogen content, since strains still showed a highly significant ($F_{(3,40)}$ = 124.73, p<0.001) difference. Although there was no difference in tissue nitrogen accumulation between strains ORS302 and CP279, the latter gave a relatively high total shoot nitrogen content of 907 mg (AYB2) and 681 mg (AYB1) compared to 784 mg (AYB2) and 705 mg (AYB1) for strain ORS302 (Table 7.4). The landraces did not differ significantly from each other, but a significant ($F_{(3,40)}$ = 3.93, p<0.05) interaction between strains and landraces detected suggests some genotypic difference in landrace/rhizobial strain association. Although, due to its large dry matter yield, AYB2 had larger total

tissue nitrogen than AYB1 with strains ORS302 and CP279, both landraces performed equally in terms of proportion (%) of nitrogen derived from the atmosphere with over 97% of their nitrogen obtained through symbiosis (Table 7.4).

Table 7.4 Mean nitrogen fixation [tissue nitrogen content (%), total shoot nitrogen content (mg) and fixed nitrogen (mg)] of AYB landraces inoculated with different strains of rhizobia. (n=6)

Treatment (rhizobial strain)	Tissue nitrogen content (% of dry weight)		Total shoot N content (mg)		*Fixed nitrogen (mg) per shoot	
	AYB1	AYB2	AYB1	AYB2	AYB1	AYB2
Uninoculated	1.22	1.18	19	22	-	-
ORS302	3.00	3.02	705	784	686 (97.3)	762 (97.2)
CP279	3.18	3.05	681	907	662 (97.2)	885 (97.6)
NGR234	1.38	2.13	23	105	4 (17.4)	83 (79.1)
s.e.d.	0.205		75.3		-	
d.f.	40		40		-	
Significance (5%)						
Landrace	0.148		0.013		-	
Rhizobial strain	<0.001		<0.001		-	
Interaction	0.015		0.221		-	

* Number in parenthesis denotes % nitrogen derived from atmosphere

7.4.2.3 Plant growth

Plant growth parameters showed that successful nodulation with strains ORS302 and CP279 contributed immensely to plant growth compared to strain NGR234 and the control treatment (uninoculated plants) (Table 7.5; Plate 7.2). Contribution to the growth of the two landraces by the strains was found to be very substantial ranging from 97.5 to 1547.4% increase in shoot dry weight (Table 7.5). There was also a significant difference in shoot dry weight ($F_{(1,40)}$ = 13.61, p<0.001) between landraces and between strains ($F_{(3,40)}$ = 157.00, p<0.001). Although AYB1 did not perform any better than AYB2 following inoculation with all the strains, the significant ($F_{(3,40)}$ = 157.00, p<0.001) interaction observed between landrace and strain suggested a genotypic difference in response of landrace to rhizobial strain. Shoot dry weight of AYB1 was greater (23.4 g) with strain ORS302 than with the other strains, while AYB2 achieved its greatest shoot dry weight (29.7 g) with strain CP279. As dry matter accumulation revealed, inoculation contributed substantially to vegetative growth of AYB landraces and this was also revealed by the difference

in leaf area of the inoculated and uninoculated plants (Figure 7.1). Strain CP279 produced a larger leaf area for both landraces than strain ORS302, while there was no significant difference between plants from the control and strain NGR234. Plants from the control and those inoculated with strain NGR234 were characterised by poor vegetative growth and yellowish leaves, usually associated with nitrogen deficiency (Plate 7.2).

Table 7.5 Mean plant growth [shoot dry weight (g) and shoot dry weight increase (%)] of AYB landraces inoculated with different rhizobial strains. n=6

Treatment (rhizobial strains)	**Shoot dry weight (g)**		**Shoot dry weight increase (% of control)**	
	AYB1	AYB2	AYB1	AYB2
Uninoculated	1.61	1.92	-	-
ORS302	23.40	26.01	1453.42	1354.69
CP279	20.55	29.71	1276.40	1547.40
NGR234	1.57	4.77	97.52	248.44
s.e.d.	2.071		-	
d.f.	40		-	
Significance (5%)				
Landrace	<0.001		-	
Rhizobial strain	<0.001		-	
Interaction	0.029		-	

Plate 7.2 Effect of different rhizobial strains (left to right, ORS302, CP279, NGR234) on growth of landrace AYB2. An uninoculated plant is shown far right. Bar = 20 cm.

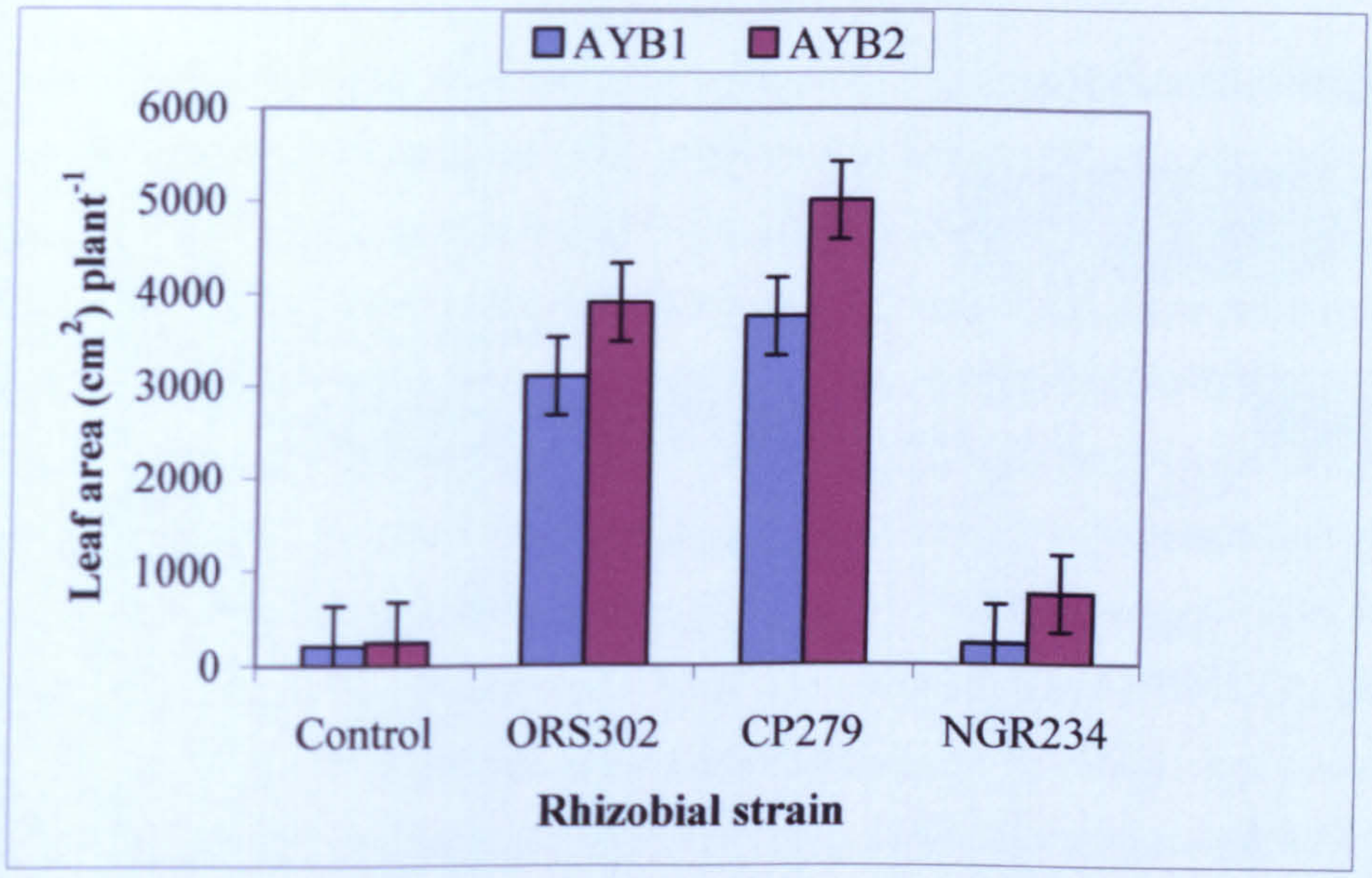

Figure 7.2 Effect of different rhizobial strain on mean leaf area of AYB1 and AYB2 landraces. Error bars = 2 *s.e.d.* (n=6)

7.5 Discussion

7.5.1 Nodulation

Results from Experiments 2 and 3 have indicated the potential of AYB as a promiscuous legume, since it was able to form nitrogen fixing nodules with 3 of the 5 strains tested and classified as both *Rhizobium* spp. and *Bradyrhizobium* spp.. The large numbers of nodules plant^{-1} (up to 365) formed on AYB landraces by some of the strains tested were comparable to that recorded in cowpea (>100) inoculated with bradyrhizobia indigenous to Ghanaian soils by Fening and Danso (2002). Cowpea was regarded as a profuse nodulator, thus indicating that AYB can be regarded as a highly profuse type. Strain ORS302, that effectively and efficiently formed a symbiosis with AYB landraces, was originally supplied from Senegal, a country in the same geographic region (West Africa) as Nigeria where the two landraces used in these experiments were collected, thus reiterating its ability to associate with various strains. In intercropping trials conducted in the sandy soils of the Benue River Basins of Nigeria to assess the effect of some food legumes used as cover crops in cassava, yam, and maize based cropping systems, Obiagwu (1995b) observed that AYB formed nodules and contributed to soil productivity and yield of the main crops without any inoculation, thus corroborating the above suggestion.

However, when inoculated with the strain (AUEB20) that has been reported by Assefa and Kleiner (1997) to induce nitrogen fixing nodules, AYB did not yield any positive results. This outcome could be attributed to several possible factors, amongst which was the quality of the strain as regards its plasmids, since the supplier indicated that the stock was very old. Another possible reason could be the genotype of AYB landraces used in this study and that used by Assefa and Kleiner (1997). Genotypic variation has been observed in this study, where the response of 2 landraces used differed from one strain to the other. Various reports have also indicated genotypic variation amongst both the microsymbionts and the hosts (Kishinevsky *et al.*, 1996; Masutha *et al.*, 1997; Robinson *et al.*, 2000). It is possible that the landraces used in this study were not compatible with strain AUEB20 and thus failed to develop nodules.

7.5.2 Nitrogen fixation of AYB landraces

All the nodule-inducing strains have been shown to be effective in nitrogen fixation. However, plants with the greatest number of nodules fixed more nitrogen than those with fewer nodules, indicating the importance of root colonisation in nitrogen fixation. This outcome is in agreement with observation on soybean by Zhang *et al.* (1997) that cultivars with a high nodule mass usually have a higher capacity to fix more nitrogen. Giller (2001) also indicated that the most obvious requirement for a legume to form an effective N_2-fixing symbiosis is the ability to form nodules that possess the necessary organisation and ancillary machinery for fixation. The reddish colouration observed on nodules and the fixation ability revealed by the ARA assay, indicated the presence of the necessary properties of an effective symbiosis. The ARA results of up to 7.04 μmoles g^{-1} h^{-1} compared to 1.02 μmoles g^{-1} h^{-1} reported by Assefa and Kleiner (1997) indicated the necessity and importance of selection/identification of a more compatible microsymbiont. Under efficient nodulation, AYB landraces obtained 79.0-97.6% of their nitrogen from the atmosphere which compared favourably to most tropical legumes mentioned by Giller (2001). This outcome reveals potential of the crop to satisfy its nitrogen needs through biological nitrogen fixation, as nodulated plants also looked healthier than the poorly nodulated and non-nodulated plants (Plate 7.2). Inoculation of legumes with an effective strain can significantly replace chemical fertilizer in supply of nitrogen.

While it is important to look at AYB's fixation capacity against other grain legumes studied elsewhere, the difference in the environment under which those observations were made puts a limit to the extent of the comparison. As Giller (2001) mentioned, fixation capacities mentioned under previous studies are only potentials in certain environmental conditions and, hence, arriving at a useful generalisation about N_2-fixing ability is difficult. The difference in tissue N accumulated by landraces suggests some underlying genotypic variation between AYB landraces. In beans (*Phaseolus vulgaris* L.), Rodriguez-Navarro (1999), observed that, although there was a difference between rhizobial strains on biomass and N concentration in shoots as a result of differences in nitrogen fixation rates, plant genotype significantly modified the performance of strains in that highly effective strains in one cultivar performed below par in another cultivar.

Since grain legumes are not only incorporated in cropping systems because of their ability to supply their own nitrogen through BNF but also contribute residual nitrogen to succeeding crops, it is important to know the amount of N fixed vis-à-vis that left behind in the field after removing the grain. Incorporation of groundnut residue, a relatively low N harvest index legume, substantially increased the yield of maize and significantly replaced the addition of urea at a rate of 75 kg ha^{-1} (Boddey *et al.*, 1997). However, it was not possible to quantify nitrogen harvest index of AYB landraces because of failure of the plants to flower and produce grain. Some grain legumes such as soybean although known to be good nitrogen fixers are also known to utilise a lot the fixed nitrogen and, as such, are unlikely to contribute much N to the next crop (Boddey *et al.*, 1997). Such legumes will not be suitable for inclusion in cropping systems commonly practised by resource-poor farmers in developing countries. Therefore, it is vital in the future to investigate nitrogen harvest index of AYB growing under conditions that allow reproductive growth in order to quantify its contribution to nitrogen fertility for the succeeding crop.

7.5.3 Plant growth of AYB landraces

Symbiotic N contribution to AYB plants has been immense with plants that received inoculum performing better than the uninoculated plants. Symbiotic effectiveness of a rhizobial population is one of the important parameters for selecting strains for inoculant production (Fening and Danso, 2002). As with nitrogen fixation, full root

colonisation by rhizobia is an important element of efficient nitrogen fixation. Increase of growth of the inoculated plants by 1547% of uninoculated plants showed that it is possible to grow AYB without the addition of any supplementary N fertilizer. However, this still needs to be investigated further under conditions allowing reproductive growth to characterise the response of the plant when it flowers and produces grain.

Although the variety of bacterial strains tested in this study was narrow, the variation observed underlines the importance for further investigations, such as screening of strains indigenous to AYB natural habitats or potential production areas and commercially available strains to formulate a composite BNF approach for the crop. Evaluation of the ability of different landraces to form effective partnership with various strains is very important, as it was clear that effectiveness of symbiosis did not only rely on the strain but also on the target landrace.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSION

8.1 Introduction

Non-conventional crops, of which AYB is one, receive very little research attention because of the high priority given to conventional crops. Such non-conventional crops are mostly neglected and under utilised hence face a threat of genetic erosion. In this study, clonal propagation protocols, characterisation of plant growth and biological nitrogen fixation were investigated with the aim of establishing basic techniques that can benefit further investigations as well as enhance production of the species as an alternative food crop. This chapter provides a general discussion of the findings and puts forward suggestions for further studies.

8.2 Morphology and growth

AYB is the most morphologically variable species in the genus (Potter, 1992). Although morphological observations were not detailed and were conducted on a narrow range of genotypes of this species, morphological variations were evident from the literature. Features such as seed colour, stem colour, internode length, leaf size and number of leaves per plant were found to be some of the differentiating characters. Growth of certain plant parts, such as root tubers, were observed only in some landraces and not in others. However, this cannot be attributed entirely to genotypic variation as other important factors controlling plant development such as environment were not investigated. Indeed, environment was found to be worthy of investigation, since some important developmental stages, such as flowering were observed only with certain temperature régimes. AYB plants grown in growth rooms at 30°C with a 12 h photoperiod as well as in the glasshouse with natural summer conditions did not flower, but flowering was observed within 7 months in plants grown in a similar room at 25°C and with the same photoperiod. Photoperiod in the tropics is relatively short and some legumes adapted to the tropics have been found to flower only at certain relatively lower temperatures, including *Phaseolus* spp. (Davies, 1997) and *Vigna subterranean* (Linnemann and Azam-Ali, 1993). The

period of development in yam bean (*Pachyrhizus* spp.) up to flowering is reported to vary with photoperiod where flower initiation is early under short days but late under long days, occurring only when photoperiod approaches 12.5 h (Sorensen *et al.*, 1993). Since photoperiodic change is concomitant with temperature, it is likely that lower temperature might play a part in *Pachyrhizus* as well as in AYB. Since tubers develop underground, it has not been possible to identify the actual time of tuber initiation, but the report that tubers are harvested about the same time as seeds (Potter, 1992) supports the possibility that tuber development follows the same pattern as that reported in yam bean (Sorensen *et al.*, 1993). In yam bean, tuber initiation occur within 4-6 weeks after establishment whatever the photothermoperiod and vegetative growth under long days is only important for shoot (photosynthetic apparatus) development required for tuber enlargement later (Sorensen *et al.*, 1993).

Vegetative growth in AYB is very vigorous with formation of numerous twining vines growing to heights >3 m. Generally, variation in growth between the landraces existed, although it was mostly not statistically significant. The difference in growth conditions between pot and soil experiments, influenced performance of the two landraces. Average growth rate was relatively slow for plants grown in pots compared to plants grown in the soil, although growth in the early stages of the soil experiment was slower than in the pot experiment. Consistent growth observed in plants grown in pots could have been due to the absence of disturbance usually associated with transplanting but was restrictive because of the pot environment. In soil, slow growth in the early stages was possibly due to transplanting shock but became fast later due to a free root run and possibly abundant mineral nutrients from basal fertilizer applied prior to planting. Different genotypes vary in their adaptation to the environment and it is likely that AYB1 performed better under restrictive pot conditions because it is more tolerant to stress than AYB2. AYB2 appeared less tolerant to stress and hence not suitable for marginal environments. Although genotypic variation in growth of AYB landraces has been confirmed, it has not been possible to identify the impact it will have on reproductive growth as the plants did not flower during these experiments.

8.3 Development of clonal propagation protocols/techniques

If genetic variation within some plant populations influences the effect of specific treatments, then it is essential to be able to work with clonal material. In this study, clonal propagation protocols were developed using nodal explants/propagules to reproduce material from existing shoot meristems with high genetic uniformity.

8.3.1 *In vitro* propagation/micropropagation (axillary buds)

Shoot regeneration from nodal explants can be used to achieve mass propagation of clonal progeny by either allowing growth of multiple shoots or a single enlongated shoot bearing discrete and separated nodes that can be cut into nodal explants and further subcultured to obtain more shoots. Tissue growth and regeneration is obtained through the utilisation of well-developed *in vitro* protocols that provide optimal conditions for physiological functions of explants. *In vitro* propagation can be divided in four stages, specifically, stage 1 (establishment of axenic and still cultures), stage 2 (proliferation or multiplication), stage 3 (production of cuttings or plantlets/root formation) and stage 4 (reestablishment in the glasshouse) (Debergh and Read, 1991). The listing of the four stages above is not intended to denigrate the initial stage of preparing or raising of stock plants under hygienic conditions limiting contamination problems. Each stage usually receives specific treatments to achieve the desired goals. *In vitro* propagation of AYB was also found to more or less follow this pattern.

Axenic cultures (>97% contamination free cultures) was achieved by surface sterilization of AYB explants through the use of Domestos bleach, although following insect infestation it was difficult to achieve the same proportion of contamination-free cultures. The climbing habit has enabled AYB to grow away from the ground where it could have been in contact with soil-borne contaminants. Axenic shoot cultures were achieved from stem nodal segments using cytokinins in different culture media, and shoot multiplication for mass propagation from such axenic explants was investigated using various media salts and media formulations. Experiments were carried out to identify the most suitable protocol to generate more shoots from axenic explants. Shoots generated in culture have been rooted *in vitro* when exposed to auxins (IBA and NAA) and successfully established in compost.

There are explants that produce enough hormones not to warrant addition of any extra regulators in the medium, and the exogenous regulator requirement, if it exists, depends strongly on the genotype and endogenous hormone content (Pierik, 1987) Cytokinin in the culture medium was required to stimulate growth from nodal explants and the AYB cultures grown on media devoid of cytokinins did not support any significant growth. Shoot growth was responsive to BAP concentration. At high BAP concentration (2.5 mg l^{-1}), more shoots were produced whereas at lower concentrations (0.25-1.0 mg l^{-1}) the shoots produced were longer. Increase in shoot number with BAP concentration, with their elongation being inversely proportional to BAP concentration, is in agreement with the observations made by Polisetty *et al.* (1997) in chickpea (*Cicer arietinum*) and Girija *et al.* (1999) in *Crossandra infundibuliformis*. The addition of BAP to media that favoured callus proliferation, regardless of the concentration, contradicts the report on the significant increase in callus proliferation of hypocotyls of adzuki bean (*Vigna angularis*) only when the BA level was increased twofold (Avenido and Hattori, 2000).

Although the performance of explants differed between establishment cultures and subcultures, the response to various culture conditions followed a similar trend. Excessive callus proliferation in the cultures still occurred at the expense of shoot proliferation and growth, but was more pronounced with subculture, resulting in the production of shoots too small for rooting. When BAP was tested alongside 2iP and TDZ, larger numbers of shoots were produced on media containing TDZ followed by BAP, although the trend changed with decrease in cytokinin concentration. In contrast, the number of shoots produced increased with increase in concentration of 2iP. Shoots were longer on media containing BAP than with TDZ and 2iP. All of the cytokinins promoted callus proliferation, although to a lesser degree with 2iP. Callus production was found to be dependent on the concentration with TDZ, but not with BAP and 2iP. Naik *et al.* (1999) attributed this same action of TDZ on pomegranate (*Punica granatum*) to its high activity that can also be inhibitory to shoot development. While cytokinin enriched media promoted callus proliferation, growth was not supported by media devoid of cytokinins, confirming the necessity for cytokinins in *in vitro* shoot growth. However, BAP has been observed to be a better cytokinin for shoot development as well as for induction (Yang and Read,

1996; Brar *et al.*, 1997; Pattnaik and Chand, 1997; Tavares *et al.*, 1998; Chitra and Padmaja., 1999). In contrast, 2iP is less active and needs to be used at higher concentration to compensate for this difference.

An ideal tissue culture medium for maximum growth should provide the tissues with sufficient essential nutrients for the duration of the culture so that the depletion of such nutrients does not limit growth. Manipulation of nutrient content in the culture medium has been shown to give varying results. The three most commonly used media [MS medium (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) and Woody Plant Medium (McCown and Lloyd, 1981)] evaluated gave different results. MS was found to be more suitable than the other two in terms of shoot size (fresh weight) and shoot number. However, there was also a significant difference between the amounts of callus produced on the three media, with more callus produced on MS than on the other media. MS medium contains the highest concentration of nutrients amongst the three and could have influenced this outcome. Most plants react positively to MS medium (Pierik, 1987) and AYB seems to be amongst such species.

As MS medium was found to be suitable for the establishment of axenic cultures of AYB, adjustment of the MS salt concentration (full strength MS and half strength MS) with and without cytokinin (BAP) was compared on shoot proliferation and growth from axenic explants. Although full strength MS with BAP produced more callus, the presence of callus was attributed to cytokinin because callus proliferation was still observed on half strength MS containing BAP but less on BAP-free MS media formulations. Shoot number, shoot height and number of nodes produced in culture were also enhanced by full strength MS and BAP than other formulations. Since reduction of MS strength affected growth of shoots, this suggests that full strength MS should be used as the basal medium and future work should focus on optimisation of cytokinin concentrations.

Auxin polar transport inhibitors prevent the transport of endogenous IAA to regeneration sites, so that an auxin/cytokinin balance becomes more favourable for the regeneration of shoot buds (Charriere and Hahne, 1998; Nakano *et al.*, 2000). Auxins usually promote callus growth (George and Sherrington, 1984), thus it may be possible to suppress callus growth in AYB cultures by using auxin polar transport

inhibitors such as 2,3,5-triiodobenzoic acid (TIBA). However, it was observed in this study that axenic explants treated with TIBA continued to produce a considerable amount of callus and there was little benefit in terms of shoot proliferation and growth. This contradicts the theory that TIBA may inhibit the transport of endogenous IAA to regeneration sites, so that an auxin/cytokinin balance becomes more favourable for the regeneration of shoot buds (Nakano *et al.*, 2000). TIBA had no beneficial effect on shoot growth and callus proliferation of two AYB genotypes.

Many researchers have recorded a synergistic effect of BAP and GA_3 on the growth of shoots *in vitro* from nodal explants (Purohit and Singhvi, 1998; Vengadesan *et al.*, 2002; Chitra and Padmaja, 2002) but this has not been the case with AYB. The initial experiment with axenic explants in the present study indicated some benefit from the addition of GA_3, with considerable elongation of shoots initiated in medium containing cytokinins although this was still accompanied by excessive callus proliferation. This suggested some form of synergistic effect of BAP and GA_3 as reported in other species. However, subsequent experiments did not confirm a clear benefit of enhanced shoot growth or reduced callus growth from media containing BAP with GA_3. This outcome suggests no benefit to AYB *in vitro* growth from the addition of GA_3.

The ultimate goal in *in vitro* propagation is to establish regenerated plants in the field and this is achieved by ensuring that *in vitro*-derived shoots develop roots for anchorage, water and nutrient uptake. Some species easily form adventitious roots on shoots produced *in vitro*, while in other it may be necessary to apply specific treatments for rooting (George and Sherrington, 1984). The trials carried out to evaluate commonly used auxins for the rooting of *in vitro* shoots of AYB revealed that rooting of AYB *in vitro* was very erratic, however, a requirement for exogenous auxin was established. IBA induced more shoots to root than NAA and 2.0 mg l^{-1} was better than 1.0 and 1.5 mg l^{-1}. Many authors report IBA as the preferred auxin for rooting most plants (Geetha *et al.*, 1997a,b; Girija *et al.*, 1999), usually with relatively low concentrations.

Gibberellins enhance shoot extension but inhibit adventitious root formation (George and Sherrington, 1984; Pierik, 1987). Addition to the culture medium of substances that block biosysnthesis of GA has been reported to give positive effects on rooting for some plants (Mckinless and Alderson, 1993). For root formation of AYB, no benefit was derived from addition of the anti-gibberellin PP333 at either the shoot initiation stage or root formation stage. Instead, there was a marked retardation in the growth of shoots when PP333 was applied at the shoot growth stage. It is concluded that the synergistic effect of auxin and the growth retardant PP333 observed in other plants was not beneficial in AYB.

Plants derived from *in vitro* culture were able to acclimatise in compost and grow into normal plants. Although a trial was not conducted in acclimation of plants due to a limited number of rooted shoots, the size of shoots seemed to play a major role as larger plants with more leaves survived compared to the relatively smaller ones. Large plants survived possibly because they had sufficient reserves to sustain them through the transition from heterotrophic to autotrophic nutrition. Transplanted strawberry plantlets were also thought to depend on stored carbohydrates within their tissues for growth and development in the early period following transplanting prior to emergence of new photosynthetically competent leaves (Grout and Millam, 1985). Since it was not determined whether the *in vitro*-derived leaves of AYB were persistent or not, it is also possible that large plants survived because of a higher photosynthetic activity furnished by their larger leaf area. The benefit of large shoots in acclimation has also been observed when large plants which were cultured in the absence of PP333 survived *ex vivo* while those retarded and deformed by because of PP333 failed.

8.3.2 Propagation from nodal stem cuttings

While tissue culture is possible for the mass production of plants, its overall costs limit its application and, therefore, there is a need to develop a more affordable alternative technology. The propagation of plants from stem cuttings is one such approach. Experiments carried out to determine the necessity of an auxin and a suitable auxin concentration for the rooting of stem cuttings of AYB proved that AYB cuttings root readily without auxin. Leafy single node stem cuttings formed roots without auxin and high auxin concentrations were detrimental, causing

mortality of cuttings, retarded buds/shoots and shorter roots. While cuttings rooted successfully without auxins (up-to 100%), root formation was more synchronised in cuttings treated with low concentrations of auxin (1.0-5.0 g l^{-1} IBA) and auxin also stimulated a larger number of roots per cutting. These findings are in agreement with other studies that found application of exogenous auxin to hasten the rate of rooting, increased root number of most species although, in some cases, relatively higher concentrations resulted in inhibited root formation and growth, reduced bud/shoot growth and even lead to mortality (Badji *et al.*, 1991; Demeke *et al.*, 1992; Edson *et al.*, 1994; Ofori *et al.*, 1996; Shiembo *et al.*, 1996; Mesen *et al.*, 1997; De Andres *et al.*, 1999; Copes and Mandel, 2000; Fett-Neto *et al.*, 2001; Aminah, 2003; Ercisli *et al.*, 2003). The ease of rooting AYB cuttings and even layered stems raises a question of why rooting *in vitro* was found to be difficult. The residual cytokinins inherent in *in vitro* derived shoots, the culture environment such as aeration of the culture medium and other factors could be playing a part in this paradox.

Carbohydrate reserves or photosynthetic capacity of plant tissues are necessary for the production of adventitious roots (George and Sherrington, 1984). AYB stem nodal cuttings possess different leaf sizes depending on their position on the vine. Initially, larger stem cuttings were used instead of smaller ones in the other experiments because it was thought they have enough carbohydrates reserve or better photosynthetic capacity to support rooting. However, rooting of AYB did not depend on leaf size as successful rooting of cuttings with different leaf sizes was achieved. Nketiah *et al.* (1998) recorded better rooting of *Triplochiton scleroxylon* K. Schum from cuttings with a larger leaf area than smaller cuttings. In contrast, AYB propagules of different sizes can be used, thus avoiding wastage of parent plant material.

8.4 Nodulation and nitrogen fixation

Nitrogen is the most limiting nutrient for plant growth and the most deficient in soils although it makes up about 80% of the earth's atmospheric air. Soil constraints such nitrogen deficiency are recognised to contribute to low crop yields in Africa (Dakora and Keya, 1997). A cheap nitrogen source exists in biological nitrogen fixation because legumes are known for their ability to fix atmospheric nitrogen through root

nodules developed in association with some strains of Gram negative bacteria of the *Rhizobiaceae.*

An evaluation of five different strains of *Rhizobium, i.e.* ORS302, CP279, NGR234, ANU240 and AUEB20, confirmed that AYB can derive adequate nitrogen through atmospheric fixation. AYB was found to be a profuse nodulator with 3 of the rhizobial strains (ORS302, CP279 and NGR 234) able to form nitrogen fixing nodules, although strain AUEB20 failed to induce nodulation contrary to the report by Assefa and Kleiner (1997). The nodulating strains varied in their infectivity (nodule number formed) as well as nitrogen fixation efficiency per nodule mass. Efficient nodulating strains also contributed to the overall growth of the plants. AYB landraces obtained 79.0-97.6% of their nitrogen from the atmosphere and increased growth of the inoculated plants by up to 1547%. These results compare favourably to most tropical grain legumes mentioned by Giller (2001) and show that it is possible to grow AYB without the addition of any supplementary N fertilizer if inoculated with compatible rhizobial strains.

8.5 Conclusions

Genotypic variation exists in morphology and growth of AYB landraces. Seed colour, stem colour, internode length, leaf size and leaf number per plant are some of the delimiting characters of AYB landraces. AYB development patterns such as flowering are likely to be photothermally controlled. Growth varies between landraces and environmental response is likely to be genotype dependent.

AYB is amenable to clonal propagation by both micro and macro means. Although clonal propagation protocols cannot be adapted to replace seed in large production systems because of their cost, they still have a role to play in providing material for research purposes to enhance the status of AYB as an alternative food crop.

In vitro propagation of AYB from nodal segments can follow two patterns; a 3 stage direct caulogenesis approach (production of micro-cuttings, induction of adventitious roots and re-establishment in the greenhouse) from initial cultures and a 4 stage approach characterised by establishment of axenic cultures, multiplication of shoots

from axenic cultures (subculture for caulogensis or further multiple shoot induction), adventitious root induction and re-establishment in the glasshouse. Explants from AYB plants grown with support respond positively to surface sterilisation with domestic bleach, however, it is imperative to keep stock plants free of diseases and pests to avoid internal contaminants which are difficult to eliminate. Cytokinins are necessary to stimulate *in vitro* growth of shoots at the stages of axenic culture establishment and shoot multiplication. BAP is a more suitable cytokinin than TDZ and 2iP. The contradictory response to cytokinin concentration between shoot proliferation and elongation suggests a 2 step approach for shoot multiplication where separate media are used; one high and the other low in cytokinin for multiple shoot proliferation and elongation respectively. Other PGRs such as GA_3 and TIBA were not beneficial to *in vitro* regeneration of AYB. MS used in its full strength is the most suitable growth medium for AYB regeneration. Auxins are necessary for the induction of roots and IBA was found to be the most suitable auxin. The benefits of anti-gibberellin PP333 on rooting observed in other plants were not observed with AYB.

AYB readily forms adventitious roots in the absence of auxin. Plants are ready for transplanting into pots within 2 weeks of root induction. However, auxin used at low concentration can improve uniformity of rooting and increase number of roots per cutting whereas high auxin concentration causes mortality of cuttings, reduces root growth and retards shoot bud development. Leaf area/size does not affect rooting ability of AYB plants and cuttings of different sizes can successfully serve as propagules. Where researchers need to work with clones and are constrained financially to utilise *in vitro* protocols for mass production, propagation of AYB by stem cuttings is recommended as a low cost technology and hence appropriate for use.

AYB is a legume that nodulates profusely with a wide range of both slow growing *Bradyrhizobium* spp. and fast growing *Rhizobium* spp. Therefore, there exists a cheaper alternative source of nitrogen for AYB in the form of biological nitrogen fixation and growers of crop should be encouraged to utilise the technology in order to minimise production costs.

The work reported in this thesis will add impetus to endeavours to enhance the potential of African yam bean as an alternative food crop. The clonal propagation techniques investigated have shown that large amounts of seed are not required to carry out experiments as the crop is amenable to vegetative propagation from a limited source of plant material. This has made it possible to investigate important areas such as morphology/growth and nitrogen fixation of the crop. These studies were carried out using clonal material raised through *in vitro* culture by nodal explants and rooting of single node stem cuttings, thus eliminating genetic variation in the experimental units that may influence response to treatments. The micropropagation procedure established in this study is expected to form the basis for future studies using *in vitro* techniques for the genetic improvement of AYB with respect to yield, disease and pest resistance and water relations.

8.6 Suggestions for future work

As a new or neglected crop, there are many avenues that still need to be explored in order to enhance the status of AYB as an alternative food crop. This section outlines areas related to work done in this study but which could not be covered because of time and other circumstantial constraints and outlines other areas the author feels are pertinent to enhancement of utilisation of the species.

8.6.1 Morphology and growth studies

Morphological and molecular characterisation studies of the existing landraces is required. Morphological characteristics of existing landraces need to be correlated with detailed studies using state-of-the-art molecular approaches as applied to other grain legumes (Massawe *et al.*, 2005; Phansak *et al.*, 2005). The growth and development of plants should be investigated in various environment to characterise developmental patterns (how photoperiod and temperature affect phenology), production of assimilates and partitioning of assimilates. An integrated study of the phenology and physiology of the species is required to characterise the developmental pattern that will provide guidelines for production.

8.6.2 *In vitro* studies

In vitro protocols are useful tools in the field of plant science in general, benefiting areas from genetic improvement (breeding) to germplasm conservation (cryopreservation) either as nodal segments or somatic embryos. Experiments are required to optimise protocols for propagation from nodal segments such as shoot multiplication from axenic explants (optimal concentration of cytokinins for shoot multiplication) and rooting of shoots (look into different auxin application methods such as pulse treatments and alteration of *in vitro* culture conditions). The possibility for direct embryogenesis or indirect embryogenesis from cell suspensions from either the voluntary callus or callus induced on various explants should be investigated. It is also necessary to establish protocols for anther and ovule culture for the production of haploid plants to be used in breeding or for development of varieties. There is also need for exploration of other protocols such as protoplast culture for genetic manipulation for breeding purposes.

8.6.3 Propagation by stem cuttings

Plants produced following different auxin treatments vary in quality attributes such root length, number of roots per cutting and size of shoot. It is important to determine how these attributes affect the ultimate establishment and growth of plants in the field.

8.6.4 Nodulation and nitrogen fixation

Work done in the current study was restricted to a few strains of bacteria and a restricted environment, hence there is a need to conduct detailed studies of the interaction of AYB with different rhizobial strains. There is also a need to utilise the *in vitro* culture protocols to study host/strain interactions at the micro level. Evaluation of the infectivity and effectivity of more rhizobial strains and further field evaluation of the effectiveness of symbiosis with different strains (commercial and indigenous) under different environments are required.

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