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Survival and Callus Induction in *In vitro* Cultured Tissues of *Cola nitida* (Malvales: Sterculiaceae)

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ABSTRACT: The micropropagation of *Cola nitida* (Vent.) Schott and Endlicher (Kola) by means of tissue culture was investigated to provide baseline information on the requirements for the survival and callus induction from kola tissues *in vitro.* The use of modified Murashige and Skoog (1962) (MS) medium (without Zn Cu eleements) as basal medium was found to be as appropriate as the unmodified MS medium, for explant's survival. The appropriate antioxidant technique was also established with 10mgl^{-1} ascorbic acid. Callus was induced successfully from cut surfaces (periphery) of young leaves *in vitro* on the medium supplemented with 0.5-5.0 mgl^{-1} naphtheneacetic acid (NAA), combined with 0.23 mgl^{-1} 6-benzyl-aminopurine (BAP) and on medium supplemented with 0.2-0.6 mgl^{-1} BAP, combined with 1.0 mgl^{-1} NAA. Successful callus induction was also obtained from the buds of single nodal explants cultured on MS medium supplemented with 0.1-1.0 mgl^{-1} NAA, combined with 2.3 mgl^{-1} BAP.

Key Words: *Cola nitida*; Stimulant crop; Plant growth regulator; Tissue culture; Micropropagation.

Introduction

Kola (*Cola nitida*) tree is a tree crop of tropical and subtropical countries and belongs to the genus Cola *C. nitida* is cultivated in West African countries because of the economic importance of their nuts. Kola nut is used as a masticatory and stimulant (Eijnatten, 1973). It is also used to produce several pharmaceuticle drugs, liqueurs and confectioneries (Egbe and Oladokun, 1987).

Many problems have been implicated for low production level of Kola, these include: the long juvenile period; low fruit/seed production; slow and uneven seed germination; serile interspecific hybrids (*C. nitida* and *C accuminata*); and prevalent self- and cross-incompatibility of *C. nitida* trees (Jacob, 1971). Numerous studies have been conducted on the conventional methods for investigating problems of kola propagation. Several attempts have been made to induce uniform germination in *C. nitida* (Eijanatten, 1973; Oladokun, 1985). Some stidies on the sterile F_1 hybrids of kola have been reported (Jacob and Scott-Emuakpor, 1975; Morakinyo *et al*; 1981). All vegetative methods pf propagation [cutting, budding, marcotting (aerial layering) and grafting] have been praticed on kola (Egbe and Oladokun, 1987). However, the problem of low productivity still remains largelyunsolved.

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Adebona (1992) suggested the use of tissue culture studies as a means of overcoming some of these problems. The application of *in vitro* technologies has been employed for a large variety of trees, mostly temperate species. (Kannan and Jasrai, 1996). Persley (1992) stressed the need for the application.of biotechniques to many tropical commodities with a view to resolving constraints to their productivity. However, successful application of tissue culture system depends soley on a judicious choice of variables including the explant type (Holme and Petersen, 1996); developmental stage and size (Eapen and George, 1990); growth medium, culture condition, growth regulator, among others (Brown, 1990). The optimization of such factors had led to successful callus formation and maintenance of callus cultures in many species (Xiao *et al;* 1997; Obembe *et al.*, 1999). This paper presents the requirements for the survivival and callus formation of kola tissues *in vitro.*

Materials and Methods

Sources of explant

Plant materials were collected from shoots of fully-grown kola trees located in the Biological Garden and seedlings raised in the screen-house of Botany Department, both of Obafemi Awolowo University, Ile-Ife, Nigeria. Young leaves and single node cuttings from seven day-old flushes of both sources were used as explants in this work. Preliminary investigation has shown that older or more mature plant materials from field-grown plants were not suitable because they could be too mature and be colonized by microbial epiphytes which are very difficult to eliminate in order to produce sterile explants.

The choice of *C. nitida* as the sole source of explants used for the investigation was based on its economic importance. It is of much more economic importance than *C. acuminata* (Eijnatten, 1973).

Sterilization Protocols

The use of vegetative shoot based on the fact that it serves as a ready source of explants. Young leaves and nodal explants were washed under running tap water for one hour. All other steps of the sterilization procedure were carried out under sterile conditions inside a Laminar Flow Chamber. Explants were washed in (a) 10% calcium hypochlorite (CaOC1) for 10 minutes (b) 10% CaOCl for 15 minutes adnd (c) 15% CaOCl for 10 minutes. A 20 second-pretreatment with 70% ethanol prior to CaOCl treatments were also investigated. A drop of Tween 20 per 100ml of freshly prepared calcium hypochlorite was used as the surfactant. Plant materials were rinsed four times with sterile distilled water. Leaf discs (10 mm diameter) were cut using a sterilized and flamed stainless steel cork borer. The explants were cultured on unmodified MS nedium (Leaf discs, cultured face down), and incubated at 25 ± 2°C in the dark. The implanted materials were kept under observation for 2 weeks.

Media selection.

Two nutrient media tested were MS medium and its modification used for banana and plantain meristem culture *in vitro* (Esan, personal communication). Excluded as basal salt modification of the MS medium are (i) $ZnSO_4$ $7H_2O$ at 8.6 mgl^{-1} medium and (ii) $CuSO_4$. $5H_2O$ at 0.025 mgl^{-1} medium.

Determination of appropriate antioxidant/adsorbent

The effectiveness of an adsorbent, activated charcoal (AC) and an antioxidant, ascorbic acid (AS) on the prevention of accumulation of toxic products of phenolic oxidation were compared. Various concentrations of AC; 0.1, 0.3, 0.5, 0.7 and 1.0% and 2.0, 4.0, 6.0, 8.0 and 10.0mgl^{-1} AS were added separately to each of the culture solutions of the two nutrient media prior to autoclaving. The medium without antioxidant/adsorbent served as the control. No plant growth regulator was added to any of the culture media. The agar was maintained at 0.7% and the pH was adjusted to 5.7 ± 0.1. The culture flaks

were covered with non-adsorbent cotton wool and autoclaved at 121°C and 15b/in^2 pressure for 15 minutes.

Leaf callus induction.

The basal medium used for these and subsequent experiments was the modified MS medium (without Zn and Cu elements). Four different sets of auxin/cytokinin combinations were tested. The first two sets were supplemented with either of the auxins -NAA or 2,4-dichlorophenoxyacetic acid (2,4-D), at 0.5, 1.0, 3.0, and 5.0 mgl^{-1}, combined separately with 0.23 mgl^{-1} BAP. The range of NAA concentration tested for callus induction was adopeted according to Lydiane (1983). The last two sets were supplemented with either of the cytokinins -BAP or 6-furfurylaminopuring (kinetin), at 0.2, 0.6, 1.0, and 1.5 mgl^{-1}, in combination with 1.0 mgl^{-1} NAA.

Nodal explants callus induction

The following combination of auxin and cytokinin were tested :- NAA (0.1, 0.5, 1.0 and 1.5 mgl^{-1}), keeping BAP constant at 2.3 mgl^{-1} and BAP (0.6, 1.2, 1.8 and 2.4 mgl^{-1}), keeping NAA constant at 1.0 mgl^{-1}. Cultures of both explants were kept under observation for 4 weeks for callus induction.

Statistical analysis

All experiments were repeated three times. Data on sterilization protocol, antioxidant techniques and callus induction were statistically analyzed by the SAS software using a completely randomized design and means were compared at the p=0.05 level of significance using Turkey's studentized range (HSD) test.

Results and Discussion

Sterilization protocol

No significant difference was observed among treatments which involve quick dip in 70% ethanol followed by different treatments with calcium hypochlorite in either types from the juvenile plants (Table 1). The complementary effect of the pre-treatment with 70% ethanol could have been more effective if combined with the treatment with CaOC1. However, tissues damage due to the sterilization was observed in some explants treated with either 10% CaOC1 for 15 minutes of 15% CaOC1 for 10 minutes. Subsequently, a step-wise treatment with 70% ethanol for 20 seconds followed by 10% CaOCl for 10 minutes was used for all the experiments. Significant interaction was observed between the treatments and age of the plant materials. The kola leaf tissue and nodal sections obtained from the screen house-grown seedlings responded well to treatments than explants from matured field-grown plants (Table 1). This may be largely due to the exposure of the field-grown plant to microbial spores colonies. However, there was no significant interaction between treatments and the two explants types (Table 1, data not shown). Hence, the choice of young leaves and single node cuttings obtained from kola seedlings as the explants for subsequent stages of the work.

Antioxidant techniques and medium selection

All explants in the control experiment turned after the second day. No significant difference was observed in the effectiveness of AC at 0.3-1.0% in either media (Table 2). However, explants on 0.5% and above were observed to be curling up, appeared flaccid but retained their freshness after the seventh week. As for AS, there was significant difference among the various concentrations tested. AS at 10mg1^{-1} gave the highest number of viable explant (Table 2). However there was no significant difference among treatments with respect to the two basal media. Even though there was no callus induction, most of the explants on the media 10 mg1^{-1} AS and 0.3% AC still appeared viable. AC could remove plant growth hormones and some media components from the medium alongside the harmful phenolics and carboxylic

compounds (Weatherhead 1979). Owing to the foregoing, AS at 10 $mg1^{-1}$ has been adopted as the appropriate antioxidant for preventive accumulation of phenolics in *C. nitida.* Other antioxidants like Polyvinylpyrrolidone (PVP) and ½ strength MS medium (with 1g myo-inositol), for the reduction of phenolic oxidation and prevention of necrosis of explants, respectively have been reported (Egnin *et. al.*, 1998).

Table 1: Response of explants from *C. nitida* seedlings from mature kola to sterilised treatments.

Clean explants (percentage # mean + SD)				
Sterilization Procedure	kola seedling		mature kola	
	young leaf	single note cutting	young leaf	single note cutting
10% CaOCl (10mins)	00.0+0.0c	00.0+0.0b	00.0+0.0c	00.0+0.0b
70% ethanol (20 secs)+ 10% CaOCl (10mins.)	99.0±0.6a	90.7=0.9a	55.7+2.6a	5.7+1.5a
10% CaOCl (15mins)	17.0+2.1b	15.7+2.0b	7.7+1.5c	00.0+0.0b
70% ethanol (20sec) + 10% CaOCl (15mins.)	96.7+1.2a	91.0+0.6a	56.3+2.9a	5.7+0.7a
15% CaOCl (10mins.)	19.7+0.9b	16.3+1.7b	8.3+1.2c	00.0+0.0b
70% ethanol (20 secs) + 15% CaOCl (10mins.)	96.7+0.3a	91.7+0.9a	37.7+0.9b	6.3+1.5a

Means within a column having the same letter are not statistically significant according to Turkey's studentized range (HSD) test

There was no significant difference among treatments with respect to the two basal media (Table 2). Hence, the modified MS medium was forthwith adopted for other subsequent studies. This adoption does not mean an aberration, other suitable media excluded Cu and Zn elements in their formula (Morel, 1964). This, would reduce, to some extent, the cost of medium preparation. Holme-Hansen *et. al.* (1954) also stressed that Cu and Mo are optional addenda of nutrient medium for plant growth.

Leaf callus induction

Callus induction was successful on NAA (0.0-5.0 $mg1^{-1}$) with 0.23 $mg1^{-1}$ BAP (Fig. 2). The callus obtained with a combination of NAA and BAP was creamy white in appearance Fig. 1. There was no specific trend in the effect of the different NAA concentrations tested, except that callus induction was first observed with the medium supplemented with 1.0 $mg1^{-1}$ NAA and 0.23 $mg1^{-1}$ BAP. Callus induction on leaf explants of *Artemisia absinthium* was best with medium supplemented with 0.5$mg1^{-1}$ BAP and 2.3 $mg1^{-1}$ NAA (Nin *et al.*, 1996). The callus obtained in 2, 4,-D and BAP was only observed after the 8th week as whitish sparsely distributed particles on the intact surfaces of the leaf explants. Sondahl and Sharp (1978) reported callus induction from *Coffea arabica* leaves using 2, 4-D (1.1 $mg1^{-1}$) and kinetin (4 $mg1^{-1}$). Successful callus induction on the embryos of a tree species, *Tulipa gesneriana*, on medium supplemented with 2.2 $mg1^{-1}$ 2,4-D alone has also been reported (Famelaer *et.al.* 1996). Though, the same concentration range was employed for 2, 4-D and NAA treatments in this work, satisfactory callus induction could only be achieved with NAA.

Combined effect of BAP and NAA, kinetin and NAA on callus induction on leaf explants are presented in fig.3. Callus induction was successful on medium supplemented with 0.1, 0.2 and 0.5$mg1^{-1}$ BAP. With

Figure 1: Leaf-derived callus

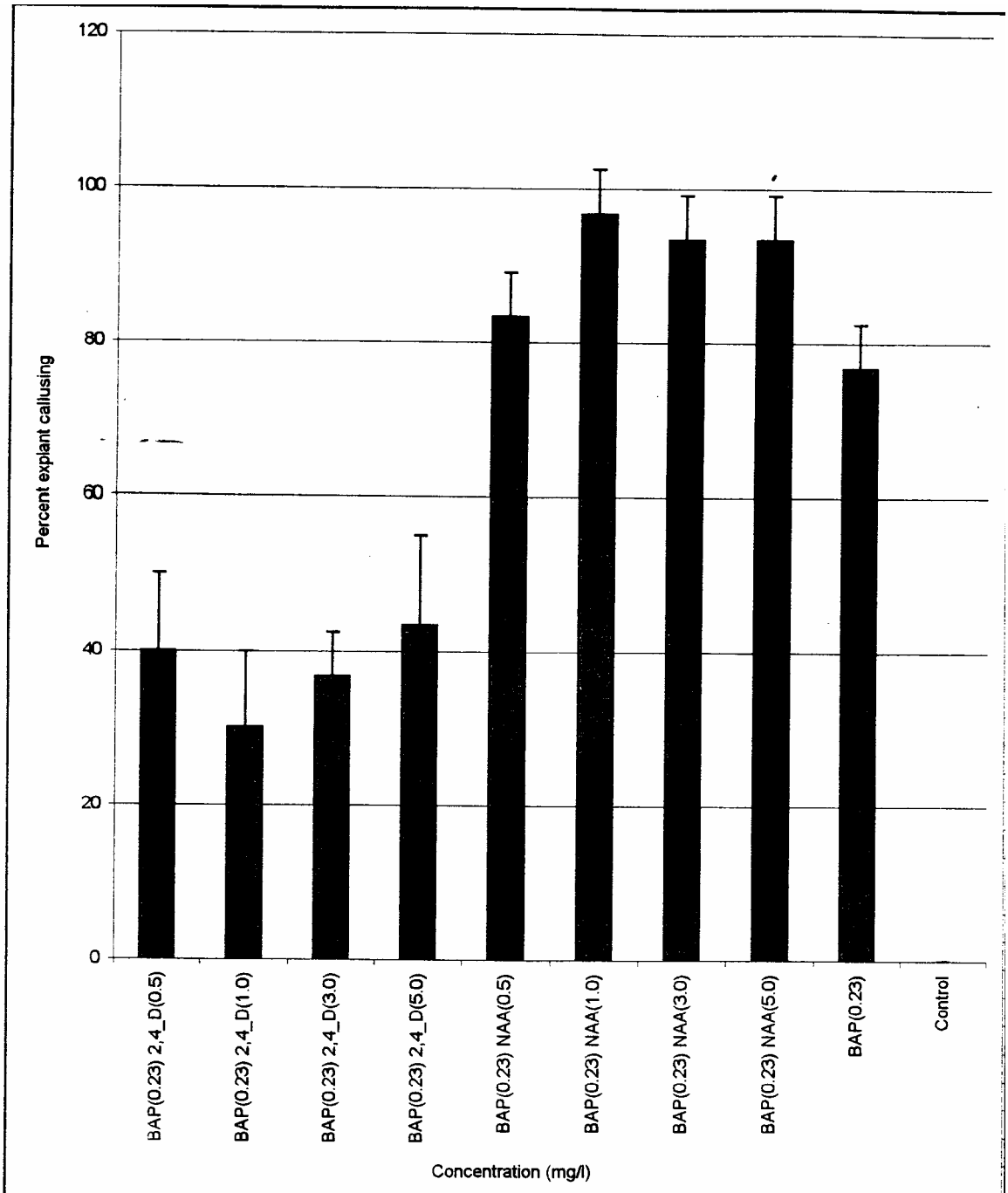

Fig. 2. Effects of plant growth regulators, naphtheneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction from kola leaf explants.
Each value is a mean ± SE from 30 explants.

kinetin however, no callus induction was observed, although the leaf cultures remained fresh after the 7th week. The retention of the green colour of the explant on kinetin could be due to cytokinin-delayed senescent effects (Salisbury and Ross, (1991).

Table 2: Effect of activated charcoal and ascorbic acid on the prevention of phenolic products on cut surfaces of kola leaf explants.

Viable explants (percentage # mean ± SD)		
Antioxidant	[1]MS medium	[2]mdMS medium
Activated charcoal		
0.00%	0.00±0.0c	0.00±0.0c
0.10%	72.3±2.5b	72.6±2.9b
0.30%	96.3±1.5a	96.0±1.0a
0.50%	96.6±1.2a	96.3±0.6a
0.70%	96.3±1.5a	96.6±1.5a
1.00%	97.6±0.6a	98.0±1.0a
Ascorbic acid		
0.0mgl^{-1}	00.0±0.0f	00.0±0.0f
2.0mgl^{-1}	49.0±1.7e	49.6±2.1e
4.0mgl^{-1}	64.3±1.5d	64.0±1.0d
6.0mgl^{-1}	70.3±2.5c	71.0±2.0c
8.0mgl^{-1}	89.3±1.2b	88.3±2.1b
10.0mgl^{-1}	98.3±5.8a	98.3±1.2a

Means under a treatment having the same letter are not statistically significant (P > 0.05) according to Turkey's studentized range (HSD) test.

[1]Murashige and Skoog (1962) basal salts mixture including vitamins

[2]Modified Murashige and Skoog (1962) medium (which lacks Cu and Zn elements)

Induction of callus nodal explants

Successful callus induction was achieved on explants implicated on 0.1-1.0 mgl^{-1} NAA in combination with 2.3 mgl^{-1} BAP (Fig. 4). However, no successful attempts of callus induction was achieved with various levels of BAP in combination with 1.0 mgl^{-1} NAA (Fig.4). Single node explants heve been found suitable for micropropagation of tree species (Kannan and Jasrai,1996). Cardoso and Oliveira (1996) reported that single node explant callus can be induced in the presence of only one auxin, 2,4-D or NAA.

Conclusion

The findings of this investigation have provided baseline information upon which future *in vitro* studies on kola can be based. In spite of the inherent accumulation of phenolics which constitutes the problem facing kola tissue culture, appropriate tissue types and appropriate package or medium requirements for their survival and callus induction have been successfully established.

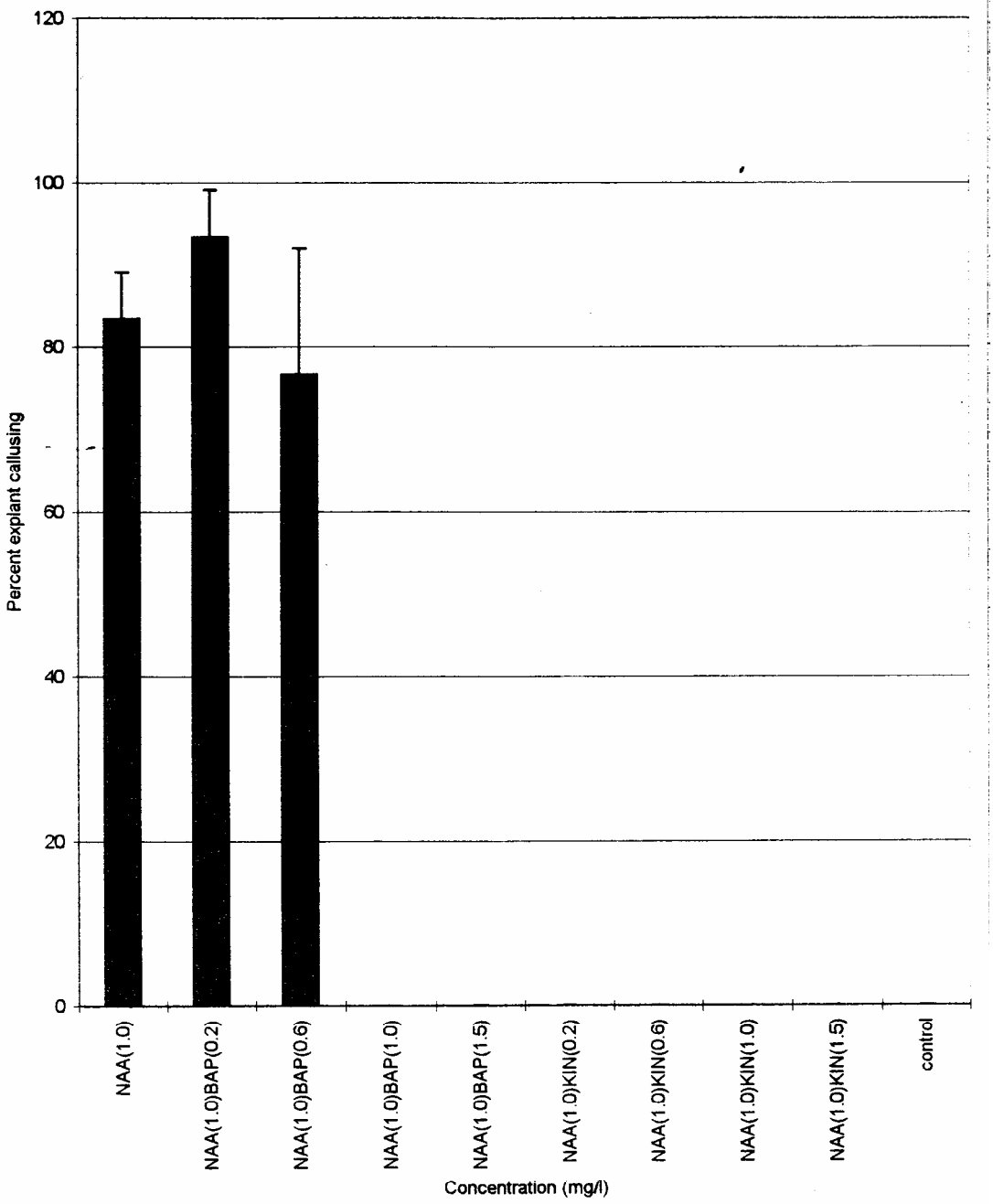

Fig. 3. Effects of 6-benzyl-aminopurine (BAP) and 6-furfurylaminopurine (Kinetin) on callus induction from kola leaf explants.
Each value is a mean ± SE from 30 explants

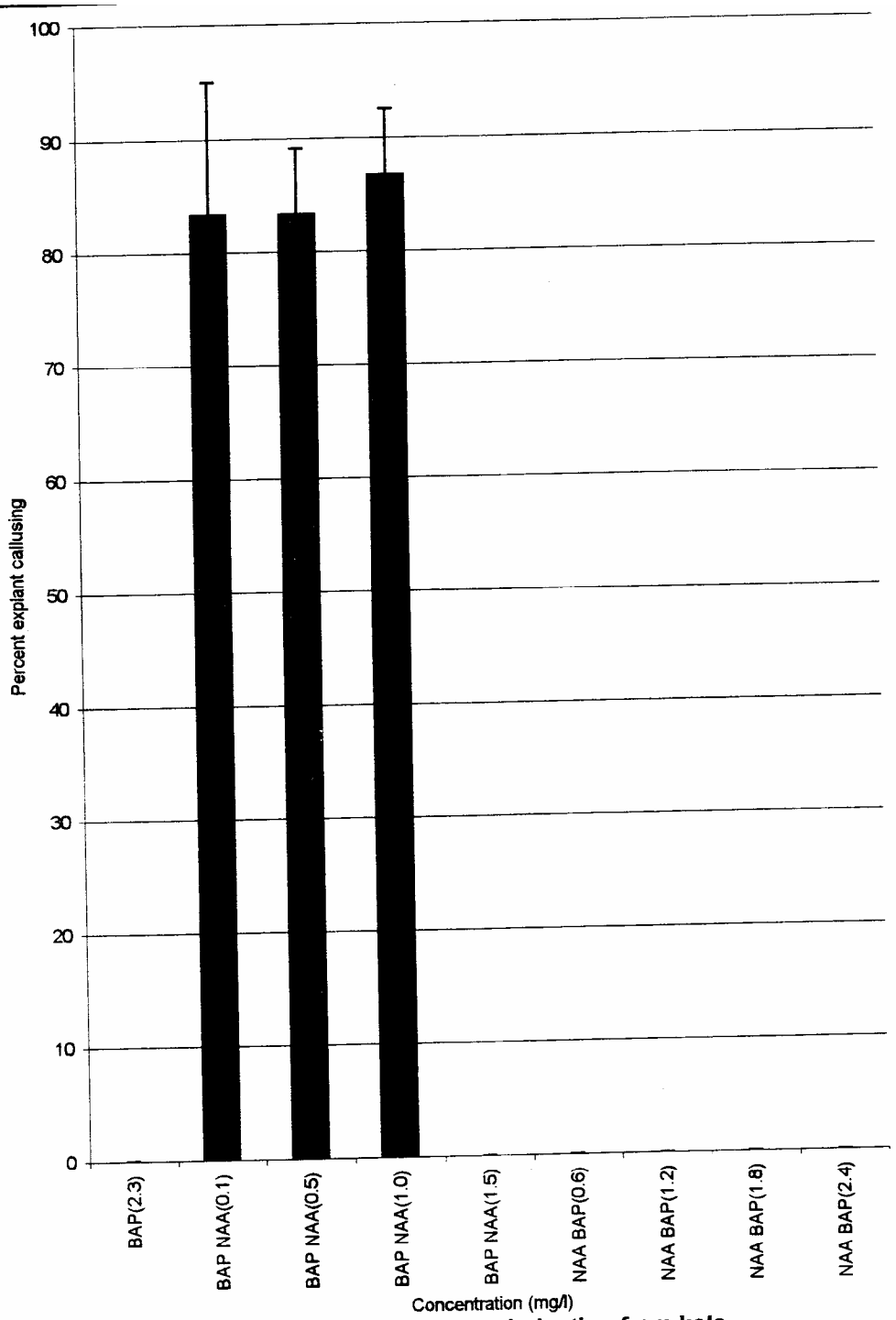

Fig. 4. Effects of NAA and BAP on callus induction from kola nodal explants.
Each value is a mean ± SE from 15 explants

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