http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences, 2 (1): 109-112, 1999 Research Article

Effects of NAA and BAP on Callus Culture and Plant Regeneration in Curly Kale (*Brassica oleraces* L.)

Shafiq Ahmad and William Spoor*
Seed Science and Technology Programme, IVARC, Islamabad, Pakistan
**IERM, Kings Buildings, University of Edinburgh, EH9 3JG, UK*

Abstract

The effect of growth regulators on Curly kale (*Brassica oleraces* L.) was studied in the Plant Growth Unit and Laboratories at Scottish Agriculture College, University of Edinburgh. Concentrations of NAA (Naphthalene acetic acid) and BAP (6-Benzyl Amino Purine) individually as well as in combination had different effects on different stages of tissue culture. In general, when ever NAA and BAP were in balance (i.e. NAA/BAP ≈ 1), callus growth was enhanced. Media supplemented with more concentration of BAP with no or low levels of NAA promoted shoot formation and reverse media type initiated roots. Maximum callus growth was observed on media supplemented with 1-1.5 mg l^{-1} each of NAA and BAP, while media supplemented with 1 mg l^{-1} of BAP only, showed best regeneration.

Introduction

Success in the technology and application of *in vitro* method is due largely, to a better understanding of nutritional requirements of cultured cells (Murashige, 1974; Gamborg *et al*., 1976; Street, 1977). Although macro and micro nutrients of *in vitro* culture media may not vary greatly from specie to specie, for successful callus yield and plant regeneration the concentrations of growth regulators (both auxin and cytokinin) are critical and are specific to genotype, explant type and need of the project. It has also been reported that each specie required a particular hormone concentration for optimum growth and differentiation (Dunwell, 1981). A study of shoot regeneration from cotyledon explants of some diploid species of *Brassica* showed species-specific responses for *in vitro* shoot regeneration (Narasimhulu and Chopra, 1987). Therefore, optimisation of the levels of growth regulators is a prerequisite for any tissue culture programme. Many workers have already reported optimisation of growth regulators for callus production in different species (Dietert *et al*., 1982; Murata and Orton, 1987; Das, 1991; Yang *et al*., 1991).

It has been well documented that many types of commercially available auxins and cytokinin can be used successfully for *in vitro* cultures of *Brassica* species (Ahmad, 1996). The literature also showed that NAA (Naphthaiono acetic Acid) and BAP (6-Benzyl Amino Purine) are commonly used auxin and cytokinin.

Hypocotyl has already proved itself as a good explant source material for successful tissue culture in curly kale (Ahmad and Spoor, 1998). Therefore, hypocotyl segments were used as explant source material to conduct this study. The level of endogenous auxin/cytokinin in hypocotyls may differ from that of the callus and these differences in the concentrations of endogenous growth regulators, may effect the requirement of these growth regulators for different material (hypocotyl, callus etc.). Therefore it is necessary to identify the appropriate hormone balance for both initiation of callus and for subsequent callus growth. The overall aim of this investigation was to find out optimum concentrations of NAA (auxin) and BAP (cytokinin) for callus induction, subsequent callus cultures and lastly for plant regeneration in curly kale.

Materials and Methods

All the culture conditions, media preparation and methodology were adopted from Ahmed and Spoor (1998). The hypocotyls were cultured on MS-media (Murashige and Skoog, 1962) supplemented with different concentrations of NAA and BAP. To provide a more comprehensive view the concentrations of NAA and BAP, from 0 to 10 mg l^{-1}, increasing with logarithmic scale, were tested first to select a narrow range. The experiment was effectively repeated to identify the optimum concentration of NAA and BAP for callus initiation and subsequent sub-cultures.

For subculturing, primary callus was harvested carefully to ensure no original explant material was included. This was then cut into small pieces (100 mg) and placed on culture media. Three callus pieces were placed in each petri dish of 50 mm diameter. Callus growth was assessed by determining callus fresh weight (Turner and Dickinson, 1993) in case of callus induction from hypocotyl segments and relative fresh weight gain (Chunren and Houli, 1991; He and Cramer, 1993) in case of subsequent callus cultures. The data was subjected to statistical analysis, through Fisher (1958) standard analysis of variance techniques (Steel and Torrie, 1980) to obtain the level of significance among different treatments.

It has been well documented that high concentrations of cytokinin along with no or very low concentration of auxin promote shoot regeneration (Ahmad, 1996). Same findings have also been observed in case of curly kale (Ahmad and Spoor, 1998). Keeping in view the previous findings the

concentration combinations of the growth regulators studied were 0/0.5, 0/1,0/2, 0/5, 0.1/0.5, 0.1/1, 0.1/2, 1/2, 1/5 mg l^{-1} of NAA/BAP. Callus was harvested and cut into small pieces (100 mg each). Callus pieces were pooled together. Culture media differing only in their growth regulators concentrations was prepared and dispensed into 60 ml specimen container. Three pieces of callus were picked randomly and placed on the medium in each container. Each combinatico of the growth regulators was considered a single treatment, and there were a total of 5 replications for each treatment. All the containers were randomized in the culture room.

The methodology of Ogihara and Tsunewaki (1979) was adopted to measure the shoot regeneration from cultures on different growth regulators concentrations. After 4 weeks of incubation, categorical data was collected.

Callus pieces were graded into 4 classes.

1. No visible differentiation
2. Only shoot bud formation
3. Shoots <1 cm
4. Shoot $\geq$ 1 cm

Number of explants with in each group was determined. Data was subjected to x^2 test to investigate the statistical differences for regeneration ability for the different types of media under study.

Results and Discussion

Callus Cultures: The hypocotyls were cultured on 16 media type differing only in their growth regulators concentrations. The concentrations of NAA and BAP used were 0, 0.1, 1 and 10 mg l^{-1} each, in all combinations. Analysis of variance (ANOVA) revealed highly significant differences among the levels of treatments, individually as well as in interaction (Table 1). Fig. 1a shows that both NAA and BAP are necessary for good callus initiation and callus growth. No callus growth was observed on the medium without growth regulator(s). The results indicated that there was a good callus growth when the NAA and BAP were in 1:1 ratio or the concentration of NAA was slightly more than that of the BAP (Fig. 1a). Maximum callus induction, observed in this experiment, was on the medium with 1 mg l^{-1} of each NAA and BAP each, which was significantly more than that on other combinations (LSD = 0.282, $p=0.05$).

Taking in consideration the results of the previous experiment the concentrations of NAA and BAP were refined. The new concentrations tested were 0.5/0.5, 1/0.5, 1/1, 1.5/1, 1.5/1.5, 2/1, 2/1.5, 2/2, 5/2 and 5/5 mg l^{-1} of NAA/BAP in combinations. Each concentration was considered as a treatment. Analysis of variance revealed highly significant differences among the treatments (Table 2). Again maximum callus growth was observed on the medium with 1/1 mg l^{-1} of NAA and BAP (Fig. 1b). Callus initiation and growth at this medium was not significantly more than that on the media supplemented with 1.5/1 and 1.5/1.5 of NAA and BAP, but significantly more than that on all other media type (LSD = 0.65, $p=0.05$). No significant differences for the callus initiation on these three concentrations revealed that the concentration of NAA and BAP from 1-1.5 mg l^{-1} each, in 1:1 or NAA concentratio slightly higher than that of BAP is optimum for callus induction from hypocotyls of curly kale.

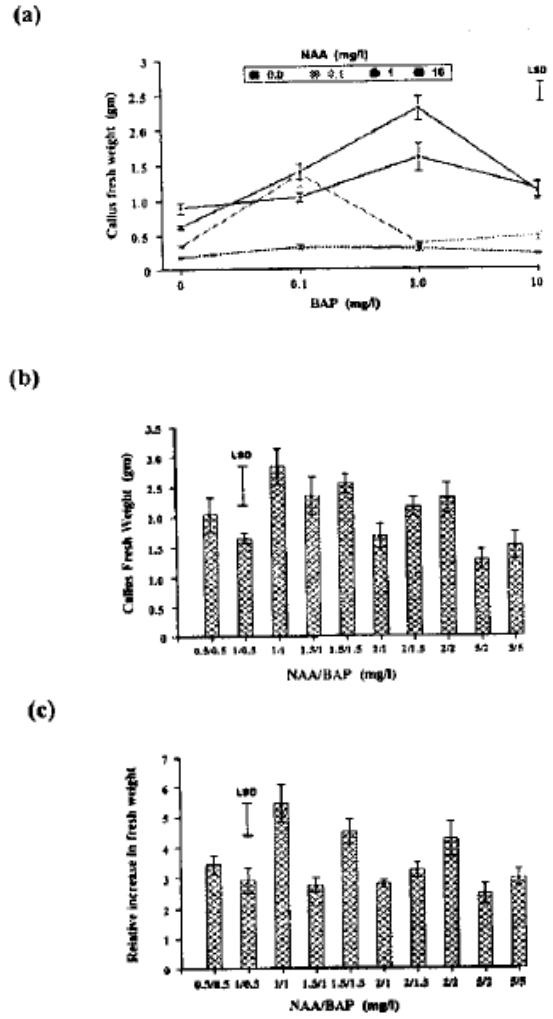

Fig. 1: Influence of different levels of NAA and BAP on callus induction and subsequent callus culture of Curly kale. Data was collected after 4 weeks of incubation. Mean SEM, n = 5

(a) Individual effect of NAA and BAP on callus initiation, using hypocotyl segments

(b) Effect of NAA and BAP, in combination, on callus initiation

(c) Effect of NAA and BAP on subsequent callus cultures

The same results were also confirmed for subsequent callus cultures (Fig. 1c). In general, these experiments revealed

Table 1: Analysis of variance of callus fresh weight from hypocotyl explants of curly kale on different levels of NAA and BAP (a). Levels of growth regulators tested, ranged from 0-10 mg l^{-1}, n = 5

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
NAA	3	15.181	5.060	102.84**
BAP	3	4.882	1.626	32.83**
Interaction	9	7.519	0.853	16.85**
Residual	64	3.172	0.050	
Total	79	30.754		

Table 2: Analysis of variance of callus fresh weight from hypocotyl explants of curly kale on different levels of NAA and BAP (b). Levels of growth regulators tested, ranged from 0.5-5 mg l^{-1}, n = 5

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatments	9	10.992	4.548	5.997**
Residual	40	10.352	0.758	
Total	49	11.344		

**Highly significant at $p<0.01$

that when even auxin and cytokinin were in balance (i.e. NAA/BAP^{-1}) there was more callus growth in general as compared with that obtained on other combinations. In the literature studied, although no one has concluded that auxin and cytokinin in balance give the best callus yield, overall a similar trend has been shown in most of the investigations dealing tissue culture in *Brassica* spp. (Sharma *et al*., 1990). The same results has also been demonstrated by other species in genus *Brassica* (Ahmad and Spoor, 1998). Flick *et al*. (1983) reported that generally a high concentration of auxin and low concentration of cytokinin in the media, promotes abundant cell proliferation with formation of callus. This report was partially supported by the current findings. In this study, although the optimum concentration for a good callus production was found on the media having auxin and cytokinin in balance (i.e. NAA/BAP ≈ 1), in general media with slightly higher concentrations of NAA as compared with that of BAP also gave satisfactory callus yield (Fig. 1a), but the roots were also emerged from the callus pieces.

Shoot Regeneration: X^2-test (= 47.87, p = 0.01, d.f = 27) of shoot regeneration ability, showed highly significant differences for shoot regeneration frequencies at different NAA/BAP concentrations. Considering the categories 3 and 4 maximum shoot formation was observed on the media with 1 mg l^{-1} of BAP only (Fig. 2). At this concentration about 24 percent of the explant pieces showed 1 cm long (grade 4) regenerated shoot. More than 80 percent of total explant pieces showed differentiation. About 60 percent of the explants showed shoot regeneration (grade 3 and 4), while the frequency of the explant pieces showing shoot formation on any other media type was not more than 40 percent.

According to Flick *et al*. (1983) generally low auxin and high cytokinin concentrations in the medium resulted in the induction of shoot morphogenesis. Lazzeri and Dunwell (1986) also reported that in *Brassica oleracea* shoot formation occurred when the cytokin:auxin ratio was greater than 1. Singh (1988) reported that lower concentrations of BAP and Kinetin (0.5 mg L^{-1}) induced roots while higher concentration (1-5 mg l^{-1}), induced shoots. Similar results have also been observed by Sharma *et al*. (1990). According to their findings the addition of BAP induced shoot buds differentiation in cotyledon culture of cultivar RIK-81-1 of *Brassica Juncea*. The highest frequency of shoot buds differentiation occurred with 5.0 pM (1 mg l^{-1}) of BAP alone. They also observed that addition of NAA in conjunction with BAP reduced caulogenic response and promoted callus formation and or rooting, which was also observed in the present study. The results of Yanmaz *et al*. (1986) indicated that in culture of cauliflower, BAP promoted shoot proliferation, and the best results were obtained from 2 mg l^{-1} of BAP. Many other workers found BAP alone or with a small amount of NAA best for shoot regeneration, among them are Singh *et al*. (1985) (Cauliflower, 5 mg BAP l^{-1}), Shahzadi *et al*. (1992) (*Brassica juncea*, 1 mg BAP 1^{-1}), Msikita and Skirvin (1989) (2 mg BAP + 0.1 mg NAA l^{-1}), Neera-Pradhan and Rajbhandry (1991) (1 ppm BAP+0.01 ppm NAA), Pawlowski (1990) (2 mg BAP l^{-1}) and Yang *et al*. (1991) (2 mg BAP +0.01 NAA l^{-1}).

Only a few reports were found to contradict these findings. Singh *et al*. (1991) reported that regeneration frequency was highest in medium containing 2 mg NAA and 1 mg BAP l^{-1} . George and Rao (1983) observed that maximum regeneration with BAP (0.2 mg l^{-1}) alone was 17 percent while with the combination of BAP and NAA, 95 percent callus formed shoots in cotyledon cultures of *Brassica juncea* var. RAI-5. Furthermore, the classical findings of Skoog and Miller (1957) that organogenesis in tissue culture is governed by the balance of auxin and cytokinin in the medium, cannot be demonstrated universally. The reasons for these contrasts in results may include differences in plant material or other growing or environmental conditions.

The conclusions drawn from these investigations were that, media composition, had an effect on different stages (e.g. callus induction, subsequent callus cultures and regeneration) of a tissue culture programme. Auxin and cytokinin both were needed for conduction. In general, auxin and cytokinin in balance favoured callus growth, while high auxin with low or no cytokinin promoted rooting and high cytokinin with low or no auxin enhanced shooting.

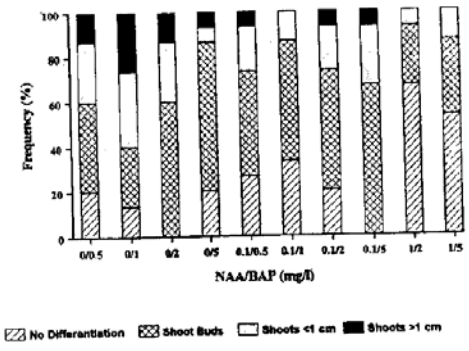

Fig. 2: Shoot regeneration frequency on different levels of NAA in combination with BAP. Source material primary callus of Curly Kale. Mean SEM, n= 5

References

Ahmad, S. and W. Spoor, 1998. Effect of genotype and expiant source on callus induction and plant regeneration in *Brassica* spp. Scient. Khyber, 1: 1-9.

Ahmad, S., 1996. *In vitro* callus selection in *Brassica* species. Ph.D. Thesis, Institute of Ecology and Resource Management, University of Edinburgh, UK.

Chunren, W. and L. Houli, 1991. Selection of rape (*Brassica napus* L.) callus cultures resistant to oxalic acid. Cruciferae Newsletter (14-15), pp: 80.

Das, R., 1991. Genotype specificity in *in vitro* callus induction of *Brassica campestris* cvs. B-54, TH-71 and B-9. Exp. Genet., 7: 72-79.

Dietert, M.F., S.A. Barron and O.C. Yoder, 1982. Effects of genotype on *in vitro* culture in the genus *Brassica*. Plant Sci. Lett., 26: 233-240.

Dunwell, J.M., 1981. *In vitro* regeneration from excised leaf discs of three *Brassica* species. J. Exp. Bot., 32: 789-799.

Fisher, R.A., 1958. Statistical Methods for Research Workers. 13th Edn., Oliver and Boyd Publ., Edinburgh, UK., Pages: 356.

Flick, C.E., D.A. Evans and W.R. Sharp, 1983. Organogenesis. In: Handbook of Plant Cell Culture, Volume 1: Techniques for Propagation and Breeding, Evans, D.A., W.R. Sharp, P.V. Ammirato and Y. Yamada (Eds.). Macmillan Publishing Company, New York, USA., pp: 13-81.

Gamborg, O.L., T. Murashige, T.A. Thorpe and I.K. Vasil, 1976. Plant tissue culture media. *In vitro* Cell. Dev. Biol.-Plant, 12: 473-478.

George, L. and P.S. Rao, 1983. Yellow-seeded variants in *in vitro* regenerants of mustard (*Brassica juncea* Coss var. Rai-5). Plant Sci. Lett., 30: 327-330.

He, T. and G.R. Cramer, 1993. Cellular responses of two rapid-cycling *Brassica* species, *B. napus* and *B. carinata*, to seawater salinity. Physiologia Plantarum, 87: 54-60.

Lazzeri, P.A. and J.M. Dunwell, 1986. *In vitro* regeneration from seedling organs of *Brassica oleracea* var. *italica* Plenck cv. green comet I. Effect of plant growth regulators. Ann. Bot., 58: 689-697.

Msikita, W. and R.M. Skirvin, 1989. *In vitro* regeneration from hypocotyl and seedling cotyledons of tronchuda (*Brassica oleracea* var. *tronchuda* Bailey). Plant Cell Tissue Org. Cult., 19: 159-165.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Murashige, T., 1974. Plant propagation through tissue cultures. Annu. Rev. Plant Physiol., 25: 135-166.

Murata, M. and T.J. Orton, 1987. Callus initiation and regeneration capacities in *Brassica* species. Plant Cell Tissue Org. Cult., 11: 111-123.

Narasimhulu, S.B. and V.L. Chopra, 1987. Plant regeneration from callus cultures of *Brassica carinata* A. Br. and its implications to improvement of oil seed *Brassicas*. Plant Breed., 99: 49-55.

Neera-Pradhan and S.B. Rajbhandary, 1991. *In vitro* culture of *Brassica oleracea* L. var *capitata* (K.K. cross). Cruciferae Newsletter (14-15), pp: 108.

Ogihara, Y. and K. Tsunewaki, 1979. Tissue culture in *Haworthia*. III. Occurrence of callus variants during subcultures and its mechanism. Jpn. J. Genet., 54: 271-293.

Pawlowski, W., 1990. *In vitro* propagation of Brassicas. Folia Horticulturae, 2: 53-61.

Shahzadi, N., Z. Ahmad, A. Hussain, M. Akram and E. Shah, 1992. Plant regeneration in mesocotyl callus of *Brassica juncea* var. Poorbiraya. Pak. J. Bot., 24: 72-78.

Sharma, K.K., S.S. Bhojwani and T.A. Thorpe, 1990. Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. Plant Sci., 66: 247-253.

Singh, N.K., A.K. Handa, P.M. Hasegawa and R.A. Bressan, 1985. Proteins associated with adaptation of cultured tobacco cells to NaCl. Plant Physiol., 79: 126-137.

Singh, S., 1988. *In vitro* plantlet from internode and inflorescence axis of *Brassica oleracea* var. *botrytis*. Curr. Sci., 57: 730-732.

Singh, S., A. Mathur and R.K. Sharma, 1991. *In vitro* studies on growth and differentiation in hypocotyl callus of *Brassica campestris* cv. yellow Sarson. J. Phytol. Res., 4: 61-66.

Skoog, F. and C.O. Miller, 1957. Chemical Regulation of Growth and Formulation in Plant Tissue Cultured *in vitro*. In: The Biological Action of Growth Substances, Porter, H.K. (Ed.). Cambridge University Press, Cambridge, UK., pp: 118-131.

Steel, R.G.D. and J.H. Torrie, 1980. Principles and Procedures of Statistics with Special Reference to Biological Sciences. McGraw Hill Book Co., Inc., New York, USA., pp: 633.

Street, H.E., 1977. Plant Tissue and Cell Culture. 2nd Edn., Blackwell, Oxford, UK., ISBN-13: 978-0520034730, Pages: 614.

Turner, A.P. and N.M. Dickinson, 1993. Copper tolerance of *Acer pseudoplatanus* L. (sycamore) in tissue culture. New Phytol., 123: 523-530.

Yang, M.Z., S.R. Jia and E.C. Pua, 1991. High frequency of plant regeneration from hypocotyl explants of *Brassica carinata* A. Br. Plant Cell Tissue Org. Cult., 24: 79-82.

Yanmaz, R., Y. Gulsen and K. Abak, 1986. *In vitro* propagation of cauliflower (*Brassica oleracea* var. *botrytis* cv. Winner osenia) by shoot tip and curd tissue culture: Influence of someauxins and BAP on shoot proliferation. Cruciferae Newsletter (11), pp: 80-89.