Phytochemistry, Vol 27, No 5, pp 1371-1374, 1988
Printed in Great Britain

0031-9422/88 $3 00+0 00
Pergamon Press plc

FORMATION OF ALKALOIDS IN SUSPENSION-CULTURED *COLCHICUM AUTUMNALE*

TAKAHISA HAYASHI,* KEIICHIRO YOSHIDA and KONOSUKE SANO

Basic Research Laboratory, Central Research Laboratories, Ajinomoto Co. Ltd, 1-1 Suzuki-cho, Kawasaki 210, Japan

(*Received* 24 *August* 1987)

Key Word Index *Colchicum autumnale*, Liliaceae, plant tissue culture, growth substances; nutritional factors; colchicine alkaloids.

Abstract—Undifferentiated callus tissues were induced from flowering shoots of *Colchicum autumnale* by treatment with 2,4-D The callus tissues producing colchicine have been grown in modified Murashige & Skoog medium containing IBA and kinetin as growth factors. The type of nitrogen source in the medium influenced alkaloid formation. Although nitrate or ammonium as the sole nitrogen source inhibited the formation of colchicine as well as growth, the formation and growth were better with 20 mM ammonium plus 40 mM nitrate. Addition of SO_4^{2-} (20 mM) markedly increased the formation of colchicine. At high concentrations, PO_4^{3-}, Ca^{2+}, and Fe^{2+} were inhibitory for formation. The identity of colchicine formed by callus was confirmed by UV and mass spectrometry analyses.

INTRODUCTION

Colchicum species produce alkaloids which have been included in many pharmacopoeias Colchicine is used in the treatment of gout and demecolcine is an antineoplastic agent. Colchicine was originally used in biological experiments to induce polyploidy or multiplication of the chromosomes in a cell nucleus [1]. Currently, the utilization of this alkaloid is increasing in the area of plant breeding; for instance, to produce either female or male sterility to make first filial generations by hybridization.

Recently, many useful secondary metabolities have been obtained in large amounts from tissue cultures of higher plants, e g shikonin from *Lithospermum erythrorhizon* [2], saponin from *Panax ginseng* [3], berberine from *Berberis wilsoniae* [4], Rosmarinic acid from *Coleus blumei* [5] The levels of the metabolites in the above cases are higher than those of intact plants. Using the same rationale, we may expect *Colchicum* cultures to be able to produce colchicine alkaloids in a fermentor tank.

The callus of *Colchicum autumnale* has already been induced and cultured in Linsmaier & Skoog [6] medium containing 2 ppm 2,4-D and 0.3 ppm kinetin as reported by Hunault [7]. However, no data were presented on the formation of alkaloids. The present communication describes the production of alkaloids in suspension-cultured *Colchicum* cells and examines the effect of growth substances and nutritional factors on the formation of colchicine. This is the first report on the production of colchicine alkaloids by plant tissue culture

RESULTS

Plant hormones

Callus tissues induced by 10^{-5} M 2,4-D and grown for two months was cultured in Murashige & Skoog [10] medium containing 0.5 μM IBA plus 0.5 μM kinetin because cell growth was inhibited by 2,4-D and levels of colchicine alkaloids markedly decreased in the medium containing 2,4-D. The calluses were transferred to solid medium containing 0.5 μM IBA plus 0.5 μM kinetin at two month intervals. The formation of colchicine and callus growth was stable in the medium containing IBA and kinetin during growth. Although IAA could be used instead of IBA as an auxin, the callus generated mostly rootlets during growth. The morphological phenomenon is similar to that of callus cultured in auxin-free medium. The results suggest that 2,4-D is required for the formation of callus from *Colchicum* tissues but represses the formation of alkaloids, and IAA is an unstable auxin, probably due to the degradation by IAA oxidase [11].

Effects of IBA and kinetin on colchicine formation and growth of suspension-cultured *Colchicum* cells were examined. Colchicine formation was stimulated by 0.5 μM kinetin, but high concentrations (5 μM) of kinetin were inhibitory (Fig. 1) IBA stimulated cell growth in the presence of kinetin. We therefore conclude that 0.5 μM kinetin plus 0 5 μM IBA is optimal for colchicine formation and cell growth.

The time course of colchicine formation in callus cultures on medium with 0.5 μM IBA plus 0.5 μM kinetin is shown in Fig 2. The formation was parallel to growth until the cessation of cell growth after seven week.

Carbohydrates

Several carbohydrates, e g. xylose, arabinose, glycerol, galactose, maltose, and cellobiose, were less effective than sucrose for growth and alkaloid formation. Glucose and fructose supported growth to a limited extent (80 and 60% of sucrose control, respectively) and lower levels of colchicine than sucrose as carbon source. The results showed that sucrose is the only effective carbon source for colchicine formation in suspension-cultured *Colchicum* cells.

*Author to whom correspondence should be addressed.

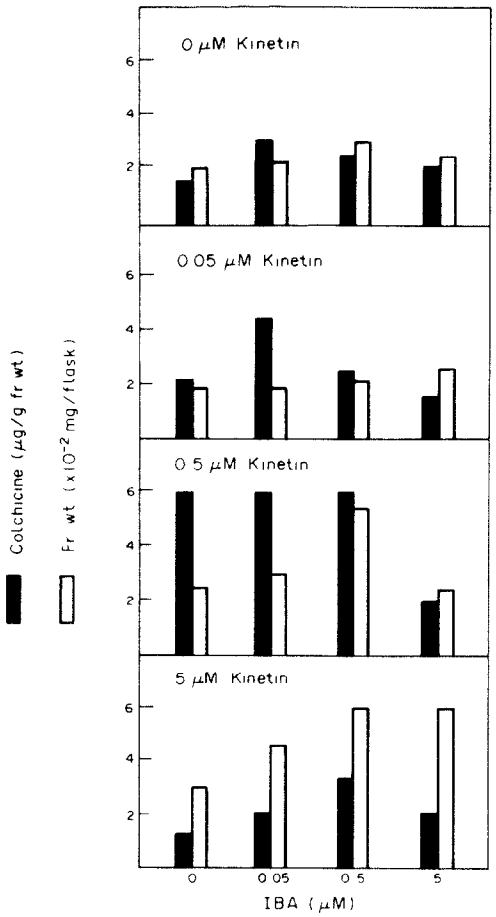

Fig 1 Effects of IBA and kinetin on colchicine formation and cell growth

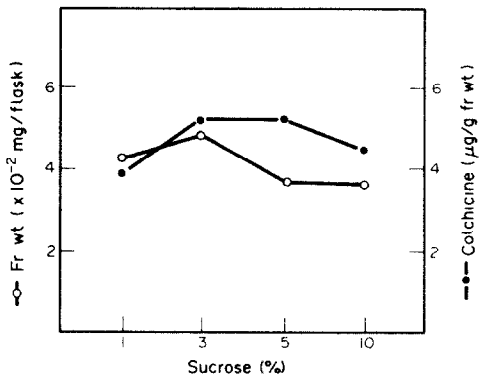

Fig 3 Effects of sucrose concentration on colchicine formation and cell growth

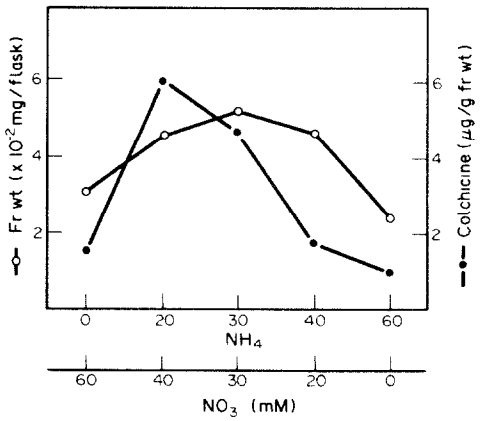

Fig 4 Effects of NH_4^+ and NO_3^- on colchicine formation and cell growth

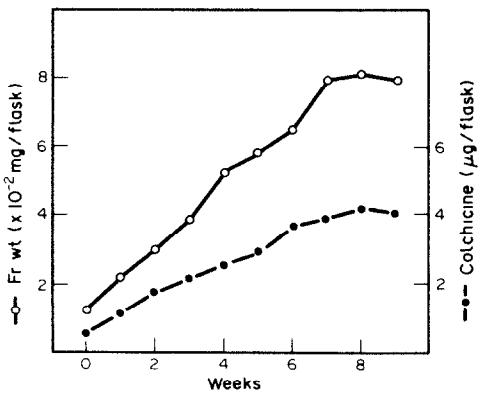

Fig 2 Time course of cell growth and colchicine formation

High concentrations of sucrose (more than 5%) were inhibitory to growth (Fig 3) Both colchicine formation and growth were highest with a 3% sucrose concentration

Nitrogen sources

The biosynthesis of shikonin derivatives is markedly inhibited by NH_4^+, while NO_3^- has no significant effect [8] Recently, Yazaki *et al.* [9] reported that the repression of shikonin production in *Lithospermum* cells cultured in a medium containing NH_4^+ is closely related to the accumulation of glutamine in high concentrations. Therefore, the effect of each nitrogen ion on the formation of colchicine alkaloids was tested The ratio of NH_4^+ and NO_3^- in the basal medium (60 mM as N) was changed without altering the concentration of nitrogen sources When NH_4^+ or NO_4^- was used as the sole nitrogen source, formation of colchicine and cell growth were markedly inhibited (Fig 4) Cells grew better in the presence of NH_4^+ and NO_3^- in the proportion of 1 1, but the formation of colchicine was inhibited at a higher ratio of NH_4^+ to that of NO_3^- The concentration (0–100 mM) of nitrogen sources in the medium was changed without altering the ratio of NH_4^+ and NO_3^- (1 2) The optimal nitrogen concentration was 60 mM for both growth and colchicine formation The results suggest that both NH_4^+ and NO_3^- are required for the formation of colchicine and growth We have concluded that 20 mM ammonium

plus 40 mM nitrate is optimal for colchicine formation and cell growth.

Inorganic ions

Sulphate ion as sodium sulphate markedly increased colchicine formation (Fig. 5). The increase was observed at a certain concentration between 20 mM and 50 mM. The effect of phosphate as potassium hydrogen phosphate (KH_2PO_4) and calcium as calcium chloride on colchicine alkaloid formation were tested. Both PO_4^{3-} and Ca^{2+} inhibited the formation at high concentrations, 2.5 mM and 10 mM, respectively (Figs 6 and 7). Mizukami *et al.* [12] reported that Fe^{2+} stimulated growth but inhibited shikonin derivative formation at high concentrations (more than 100 μM). The effect of Fe^{2+} as ion sulphate on colchicine alkaloid formation was to increase formation at 100 μM and inhibit it at higher concentrations (1 mM) (Fig. 8)

Identification of Colchicine

In order to identify the colchicine formed in suspension-cultured *Colchicium* cells, large quantities of cells were cultivated in modified Murashige & Skoog's medium modified as above and named 'Col medium' (Table 1). Ten grams of cells were extracted with 30 ml of ethanol and the extracts were analysed by HPLC on Lichrosorb RP-18 and elution with acetonitrile–methanol–water, (7.1:12) The fraction corresponding to colchicine was colleted and further purified by HPLC using gradient elution with 5% to 90% acetonitrile.

The isolated sample had an *A* maxima at 245 and 350 nm and minima at 220 and 292 nm. The sample was further subjected to mass spectrometry. The spectrum of the sample exhibited the most intense fragment peak at *m/z* 312 [13]. This is probably formed by loss of acetamide from the $[M]^+$. The ion further decomposes either by loss of a methyl group or by loss of an aromatic methoxyl group to sire peaks at *m/z* 297 and 281, respectively. The peak at *m/z* 371 is formed by the loss of carbon monoxide from the $[M]^+$. The UV and mass spectra of the isolated sample were identical to those of colchicine [13]

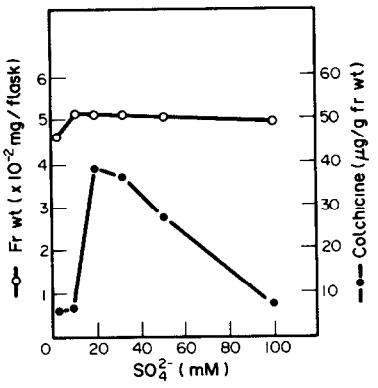

Fig 5. Effects of SO_4^{2-} on colchicine formation and cell growth

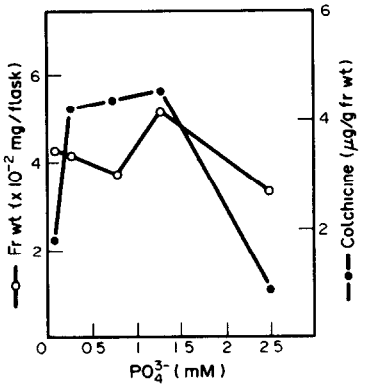

Fig 6 Effects of PO_4^{3-} on colchicine formation and cell growth

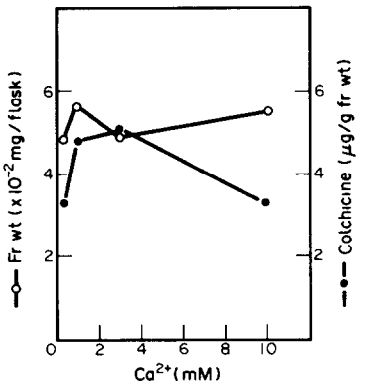

Fig 7. Effects of Ca^{2+} on colchicine formation and cell growth

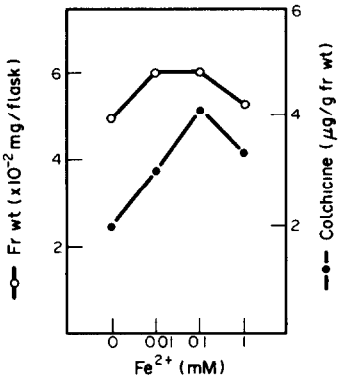

Fig. 8. Effects of Fe^{2+} on colchicine formation and cell growth

DISCUSSION

We have developed a culture medium for *C autumnale* tissues allowing production of colchicine for the first time. The medium developed is given in Table 1 as COL medium, notably, it requires high concentrations (20–50 mM) of sodium sulphate for colchicine formation (Fig. 5). Since sodium chloride did not affect the formation of

Table 1 Constituents of the tissue culture medium used for *Colchicum autumnale* (COL medium)

Constituent	mg/l	mM
IBA	0 1	0 0005
Kinetin	0 1	0 0005
Sucrose	30 000	8 8
KNO_3	1900	18 8
NH_4NO_3	1650	20 6
Na_2SO_4	2840	20
$CaCl_2\ 2H_2O$	120	3 0
KH_2PO_4	170	1 25
FeNaEDTA	36 7	0 1
$CoCl_2$	0 025	0 0001
$CuSO_4$	0 025	0 00016
H_3BO_3	6 2	0 1
KI	0 83	0 005
$MgSO_4\ 7H_2O$	370	1 5
$MnSO_4\ 4H_2O$	22 3	0 1
$Na_2MoO_4\ 2H_2O$	0 25	1 0
$ZnSO_4\ 7H_2O$	8 6	0 03
Inositol	100	0 56
Nicotinic acid	0 5	0 004
Thiamine HCl	0 1	0 0003
PyridoxineHCl	0 5	0 0025
Glycine	2 0	0 027

colchicine alkaloids, the effect was probably due to sulphate ions

We have not identified demecolcine, which is an important component of the alkaloids found in intact *Colchicum* tissues By HPLC analysis of callus extracts, the peak which corresponded to demecolcine was obtained only occasionally when high levels of colchicine accumulation were obtained The formation of demecolcine probably requires high levels of colchicine accumulation which may control the biosynthetic pathway between demecolcine and colchicine However, the amount of colchicine in our callus cultures (30–40 μg/g fr wt) is usually ten times lower than that in intact corms (240–400 μg/g fr wt) Light irradiation also did not increase the formation of alkaloids, although light may stimulate the metabolism of phenols which are involved in the biosynthesis of colchicine [14] Further studies on the production of colchicine alkaloids examine the regulation of colchicine biosynthesis [15]

EXPERIMENTAL

Cultured callus Callus tissues were derived from flower shoots of *C autumnale* cultured on Murashige & Skoog [10] basal agar medium containing 10^{-5} M 2,4-D and 10^{-6} M kinetin These tissues were transferred to the same basal medium containing 0 5 μM IBA and 0 5 μM kinetin every 2 months All cultures were maintained at 25° in the dark Before inoculation of the callus into test liquid medium, small pieces of the stock callus were transferred to the same basal liquid medium (50 ml) with no hormones in 200 ml Erlenmeyer flasks for 3 days to adapt cells to liquid medium

Culture conditions Murashige & Skoog [10] medium containing 0 5 μM IBA and 0 5 μM kinetin was used as the starting liquid medium *Ca* 125 mg fr wt of suspension-cultured callus was inoculated into a 50 ml Erlenmeyer flask containing 10 ml of test medium, after which the callus was cultured at 25° on a rotary shaker (100 cpm) in the dark for 4 week

Determination of content of colchicine alkaloids Fresh callus tissues (20–30 mg) were extracted with 100 μl of ethanol at 80° for 30 min The mixt was cooled and centrifuged at 17 000 rpm for 20 min and the supernatant (10 μl) analysed by HPLC on a LiChrosorb RP-18 (5 μm) column [16] Analysis was carried out at room temp using MeCN–MeOH–20 mM K_3PO_4 (pH 6 2) (7 1 12) A UV monitor operated at 350 nm was used as detector Alkaloid content was expressed as μg/g fr wt

Spectral analysis MS were measured with a direct sample inlet system at 220° Ionization energy was maintained at 70 eV, the ionizing current at 300 μA

Acknowledgements—We thank Dr T Furuya (Kitasato University, Japan) for his valuable advice and Dr H Flores (Louisiana State University, U S A), for stimulating discussion during the final preparation of this paper We also thank Director K Tahara of this laboratory for his guidance and encouragement and Ms M Furuya for performing the MS

REFERENCES

1 Eigsti, O J and Dustin, P Jr (1955) in *Colchicine in Agriculture, Medicine, Biology and Chemistry* Iowa State College Press, Ames, Iowa
2 Fujita, Y, Hara, Y, Suga, C and Morimoto, T (1981) *Plant Cell Reports* **1**, 61
3 Furuya, T, Yoshikawa, T, Orihara, Y and Oda, H (1983) *Plant Med* **48**, 83
4 Hinz, H and Zenk, M H (1981) *Naturwissenschaften* **67**, 620
5 Rosevear, A (1984) *Trends Biotechnol* **2**, 145
6 Linsmaier, E M and Skoog, F (1965) *Physiol Plant* **18**, 100
7 Hunault, G (1979) *Rev Cytol Biol Végét-Bot* **2**, 103
8 Fujita, Y, Hara, Y, Ogino, T and Suga, C (1981) *Plant Cell Reports* **1**, 59
9 Yazaki, K, Fukui, H, Kikuma, M and Tabata, M (1987) *Plant Cell Reports* **6**, 131
10 Murashige, T and Skoog, F (1963) *Physiol. Plant* **15**, 473
11 Hinman, R L and Lang, J (1965) *Biochemistry* **4**, 144
12 Mizukami, H, Konoshima, M and Tabata, M (1977) *Phytochemistry* **16**, 1183
13 Wilson, J M, Ohashi, M, Budzikiewicz, H, Santavy, F and Djerassi, C (1963) *Tetrahedron* **19**, 2225
14 Zimmermann, A and Hahlbrock, K (1975) *Arch Biochem Biophys* **166**, 54.
15 Yoshida, K, Hayashi, T and Sano, K (1988) *Phytochemistry* **27**, 1375
16 Yoneda, Y, Hayashi, Y, Shiroishi, M and Hayashi, S (1984) *J Fd Hygienic Soc Jpn* **25** 401