Phytochemistry. 1974. Vol. 13. pp. 321 to 324. Pergamon Press. Printed in England.

GROWTH AND BETAINE FORMATION IN *MEDICAGO SATIVA* TISSUE CULTURES

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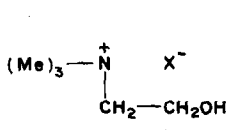
(*Revised Received* 10 *August* 1973. *Accepted* 3 *September* 1973)

Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; tissue culture; betaines; choline; stachydrine; homostachydrine; trigonelline.

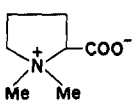
Abstract—Static and suspension tissue cultures of *Medicago sativa* were initiated from sterile seedlings, excised roots and aerial portions of the seedlings. Flask suspension cultures containing chlorophyll were also established. The various tissues were harvested after 30 days, growth rates were determined, and the tissue was later lyophilized. The lyophilized tissues from various batches were extracted separately with 70% EtOH. The betaine mixture was isolated from these extracts and from seeds of the plant as their tetraphenylborate salts. The betaines were separated from the latter complex via ion exchange and column chromatography and were identified by standard procedures. All of the tissues, regardless of origin, produced choline. Only chlorophyllous tissue biosynthesized stachydrine.

INTRODUCTION

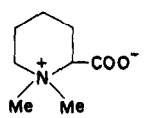
Medicago sativa L. is known to contain several betaines including choline (**1**), stachydrine (**2**), homostachydrine (**3**) and trigonelline (**4**).[1] Robertson and Marion[1] reported that seeds of the plant contain the above four betaines, while 1- to 5-month-old plants contain only choline and stachydrine. Leete *et al.*[2] found that the biogenesis of stachydrine depends not only on the presence of an amino acid precursor but also certain co-enzymes and vitamins. Trigonelline and homostachydrine which are present in corn seeds seem to disappear in the early stages of germination.[4] This investigation was conducted to determine the capacity of *M. sativa* tissue cultures to produce betaines as well as to gain more knowledge of betaine biosynthesis.



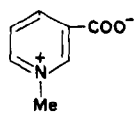
Choline (1)



Stachydrine (2)



Homostachydrine (3)



Trigonelline (4)

RESULTS

Tissues were cultured on several media in order to find ideal conditions and nutrition. While a modified White's medium was optimum for initiating callus formation, maximum growth was accomplished with the Prairie Regional Laboratory (PRL) medium.[3] Tissue

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[1] MARION, L. and ROBERTSON, A. V. (1959) *Can. J. Chem.* **37**, 1043.
[2] LEETE, E., MARION, L. and SPENCER, I. D. (1955) *J. Biol. Chem.* **214**, 71.
[3] GAMBORG, O. L. (1970) *Plant Physiol.* **45**, 352.
[4] BLAKE, C. (1954) *Am. J. Botany* **41**, 231.

which had been growing on the modified White's media exhibited a 3-fold increase in growth after transfer to PRL media.

Flask suspension cultures of aerial origin which were transferred at 30-day intervals for 22 passages showed a wet weight up to 20·1 g. Similar cultures of seedling origin had a wet weight yield up to 13·6 g.

Fermentor cultures of aerial origin exhibited a growth rate of 5·4 g/l./day while those of seedling origin were 3·6 g/l./day. Cultures on 10 ml of PRL agar media attained a wet weight of 2·5 g for the same period.

Sodium tetraphenylborate formed an insoluble complex with the betaines isolated from seeds. This complex could be separated from other precipitated materials by ethanol extraction. When this extract was applied to an anion exchange resin column and eluted with ethanol, the tetraphenylborate ion was retained by the resin and the betaine mixture eluted. This mixture could be effectively separated by cellulose column chromatography. Stachydrine was the first to be eluted followed by homostachydrine, choline and finally trigonelline. This method was rapid, convenient and much improved over the older Reineckate procedure.

All the tissue cultures produced significant amounts of choline which was initially determined by TLC. Further confirmation of choline was made via the picrate. Quantitative determination of choline showed the highest amount of choline in flask suspension cultures with chlorophyll and in tissue of root origin growing on agar media. The green tissues contained chlorophyll and xanthophyll and also stachydrine, which other tissues did not. Co-chromatography and IR analysis confirmed the presence of stachydrine. Concentrated extracts of aerial parts of normal plants showed the presence of only choline and stachydrine. Neither homostachydrine nor trigonelline was found in any tissue cultures or media.

DISCUSSION

Chlorophyll-containing tissue cultures may be metabolically different from non-chlorophyll-containing cultures.[5–7] The observation that our chlorophyllous tissue contained stachydrine is in accordance with findings of Weihler and Marion[8] who worked with normal *M. sativa* plants. Marion and Robertson[1] have reported in their biosynthetic studies of *M. sativa* that homostachydrine and trigonelline are formed in the seeds and are not actively formed in the plants until the time of seed formation. It is possible that the formation of the latter two compounds might occur in tissue cultures if the cultures were allowed to differentiate.

EXPERIMENTAL

Source of tissue and callus initiation. M. sativa seeds were obtained locally.[9] Among the media investigated for callus formation were modified White's with 15% coconut water,[10] MS tobacco medium,[11] Wood's medium,[12] and a medium originally formulated at the Prairie Regional Laboratory (PRL) at Saskatoon, Canada.[3] Seeds were surface sterilized with sodium hypochlorite solution, rinsed with sterile H_2O, placed on the surface of steril-

[5] KRIKORIAN, A. D. and STEWARD, F. C. (1969) in *Plant Physiology*, pp. 273–278, Academic Press, New York.
[6] BERGMANN, L. and BERGMANN, A. L. (1968) *Planta* **79**, 84.
[7] STEINHART, C. E. (1962) *Science* **137**, 545.
[8] WEIHLER, G. and MARION, L. (1958) *J. Biol. Chem.* **31**, 799.
[9] Felton Seed Co., Iowa City, Iowa, Certified Vernal Alfalfa, Lot V-1658.
[10] BABCOCK, P. and CAREW, D. P. (1969) *Lloydia* **25**, 209.
[11] MURASHIGE, T. and SKOOG, F. (1962) *Physiol. Plant.* **15**, 473.
[12] CAREW, D. P. (1966) *J. Pharm. Sci.* **55**, 1153.

ized 0·5% Difco agar (10 ml) and stored in the dark in an incubator at 26°. After germination the seedlings were transferred to various media listed above. Preliminary studies showed that callus growth was best initiated on the modified White's formula[10] but tissue was best maintained and exhibited maximum growth with the PRL media.[3] Therefore this was used throughout the study after callus tissue had been obtained. The modified White's formula contained 2 mg/l. 2, 4-D and 15% coconut water while the PRL medium contained 1 mg/l. 2, 4-D. It required *ca.* 5 weeks to initially obtain sufficient callus for transfer. This was designated seedling callus and was used as inocula for flask and fermentor seedling suspension cultures. Suspension cultures were initiated by aseptically transferring the callus from solid media to 500 ml flasks containing 100 ml of sterile PRL media. The flasks were placed on a rotary shaker operating at 160 rpm at 26°. Once the flask suspension cultures were established, they were maintained by transferring 1·3 g inocula to flasks of fresh media every 30 days. Tissues from flask suspension cultures were used as inocula (30 g) for stirred fermentors each containing 3 l. sterilized medium. The aeration was maintained at 800 ml/min with an agitation rate of 100 rpm at room temp.

In an attempt to induce chlorophyll formation in suspension cultures, tissues were grown in 125 ml flasks. The inoculum was from seedling callus. Each inoculated flask containing 60 ml medium was placed on a rotary shaker operating at 100 rpm. The flasks were subjected to a continuous source of light from a 30 W cool-light tube located 20 cm above the flasks.

Aerial and root callus were initiated as follows. Root portions were aseptically excised from aerial portions of sterile seedlings and each was transferred to sterile White's media to initiate callus formation. After 5 weeks incubation in the dark at 26° sufficient callus was produced for transfer to PRL media. Such callus was designated as aerial and root callus and later was used as a source for flask and fermentor cultures. All tissues were cultured for a 30-day period. At that time, a portion of tissue was used as inoculum for aerial cultures and the remainder was harvested and weighed. The harvested tissue was frozen and later lyophilized and this product was stored in amber glass bottles for subsequent chemical analysis. The agar media were frozen and stored for subsequent chemical analysis. At the time of analysis, they were thawed on a filter to separate the agar and the filtrate was concentrated under reduced pres. The concentrated liquid and agar media were stored in a refrigerator until analyzed.

Isolation of betaines from seeds and tissue cultures. M. sativa seeds (50 g), known to contain all of the betaines,[13] were ground in a Wiley mill and the product was defatted by standing overnight in 0·5 l. *n*-hexane. The hexane was decanted, the powder air-dried and successively extracted with 1 and then 0·5 l. 70% EtOH. The mixture was filtered and concentrated under reduced pressure to 300 ml. The soln was acidified with 10 ml 5% HCl. A 5% soln of sodium tetraphenylborate was added to insure complete precipitation of betaines. The ppt. was separated by filtration and shaken with 100 ml EtOH. After filtration, the ppt. was reextracted with 100 ml EtOH. The filtrates were combined and dried under reduced pressure to a residue of 0·8 g. The IRA-401S resin (50 ml) was prepared by washing with 20 ml 5% NaOH followed by an excess of dist. H_2O until eluates were no longer alkaline. The resin was then washed with 20 ml 5% HCl followed by excess H_2O. It was then equilibrated with 100 ml EtOH and slurried into a 1·5 × 40 cm column. The tetraphenylborate-betaine complex (0·8 g) was dissolved in 10 ml warm EtOH, added to the column of resin, and eluted with 200 ml EtOH. This required about 6 min. The eluate was concentrated to dryness (0·3 g). A cellulose column was prepared by making a slurry of 50 g cellulose with 200 ml $CHCL_3$–MeOH–HCl (35:4·5:0·4) which was poured into a 5 × 30 cm glass column. The tetraphynylboratebetaine complex (0·3 g) was dissolved in 5 ml the solvent mixture and added to the column with a pipet. Every 5th fraction of a total of 158 (20 ml) fractions was monitored by TLC. Modified Dragendorff's soln was used as a visualizing reagent. The same extraction and isolation procedure just described for seeds was also applied to lyophilized tissue and media concentrates with the quantities of solvents and reagents adjusted accordingly.

TABLE 1. CHOLINE IN TISSUE AND MEDIA

Type of tissue	Choline (mg/g dry tissue)	Choline (mg/ml medium)	Type of tissue	Choline (mg/g dry tissue)	Choline (mg/ml medium)
Seedling, solid	2·93	1·0	Aerial, solid	5·33	1·0
Seedling, flask	4·09	0·8	Aerial, flask	0·56	0·45
Seedling, fermentor	1·11	0·3	Aerial, Fermentor	0·27	0·45
Seedling, flask (chlorophyll)	10·76	0·0	Root, solid	10·5	0·00

Choline analysis. The method used for determining the presence of choline in tissue and media was that of Appleton.[14] The choline was extracted and then precipitated as the iodide. The latter was dissolved in ethylene

[13] SETHI, J. K. (1972) Ph.D. Thesis, University of Iowa, pp. 77.
[14] APPLETON, H. D., LA DU, B. N., JR., LEVY, B. B., STEELE, J. M. and BRODIE, B. B. (1953) *J. Biol. Chem.* **205,** 803.

dichloride and its absorbance measured at 365 nm. The choline content in tissue and media is reported in Table 1.

Chlorophyll analysis. The procedure for determining chlorophyll was that of the AOAC.[15] The final Et_2O extract in the procedure was scanned from 360 to 700 nm.

Acknowledgement—The authors thank Dr. John P. Rosazza for his assistance.

[15] ANON (1965) Assoc. Office Agric. Chemists. *Methods of Analysis*. 10 Edition, p. 114.