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Valine-Resistant Plants from in vitro Selected Tobacco Cells

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Summary. Growth inhibition of protoplasts and cells of tobacco (*Nicotiana tabacum* L.) occurs when 0.1 to 10 mM L-valine is present in the culture medium but not if L-isoleucine is simultaneously added. Mesophyll protoplasts from haploid tobacco were submitted to ultraviolet irradiation and cell colonies derived from these protoplasts were plated in a medium containing valine. Plants were regenerated from five presumptive resistant calli and retested for valine-resistance. Among the recovered plants two were definitely resistant to valine. This trait was transmitted in sexual crosses as a mendelian character.

Introduction

In vitro plant cell cultures has proved to be a useful tool for the isolation of different biochemical mutants (for recent reviews see Maliga, 1976a, b; Widholm, 1977); in particular, the selection of plant cell lines resistant to various toxic analogs of amino acids has been described by several authors (e.g.; Widholm, 1972a, b, 1974a, 1976; Carlson, 1973; Palmer and Widholm, 1975). Certain protein amino acids have also been shown to inhibit growth of various plant materials. In the latter, growth inhibition was ascribed either (i) to the inhibition of nitrate or ammonium assimilation (Filner, 1966; Heimer and Filner, 1970; Behrend and Mateles, 1975) or (ii) to a possible interference with the regulation of metabolism of related amino acids (Sandstedt and Skoog, 1960; Street, 1966; Miflin, 1969; Henke et al., 1974; Green and Phillips, 1974; Bourgin, 1976).

Growth inhibition by valine, previously observed by Sandstedt and Skoog (1960) on tobacco tissue in vitro, was recently demonstrated in tobacco protoplasts (Bourgin, 1976). This inhibition can be specifically relieved by the simultaneous addition of isoleucine, suggesting that it may be due to interference in the regulation of isoleucine biosynthesis. A similar phenomenon has also been reported in a mutant of *Arabidopsis* by Acedo and Rédei (1976).

The present study was undertaken in order to obtain valine-resistant tobacco cell lines, which might prove of value both in studies on the regulation of amino acid biosynthesis, and in assessing the efficiency of mutagenic and selective procedures on in vitro cultured plant cells.

Materials and Methods

A haploid androgenetic plant of *Nicotiana tabacum* cv. Xanthi was vegetatively propagated from cuttings in vitro (Bourgin and Missonier, 1973). After rooting, the plants were transferred to the greenhouse and cultured under conditions reported previously (Chupeau et al., 1974). Mesophyll protoplasts were prepared according to a one-step enzymatic procedure (Chupeau et al., 1974) and cultured in 10 ml of liquid medium T_0 (Bourgin and Missonier, 1978) in glass Petri dishes (90 × 15 mm), at a concentration of 10^5 protoplasts by ml.

For the mutagenic treatment, a portion of the protoplast population was submitted to ultraviolet irradiation from a germicidal lamp (G30T8), at an incident dose rate of 33 ergs. $mm^{-2} \cdot s^{-1}$, to a final dose of approximately 1000 ergs $\cdot mm^{-2}$. This treatment reduced the plating efficiency to 40–60% of that of the control population which ranged from 40 to 80%. Since it had been observed previously that the UV-irradiated culture medium was not inhibitory to the growth of protoplasts (unpublished results), the culture medium was not changed after treatment.

Both control and UV-treated protoplasts were cultured in the dark for at least 6 days before being transferred to light (2000 lux). This period of culture in the dark was intended to avoid the effects of photoreactivation on the UV-treated protoplasts (Eapen, 1976; unpublished results) and the depressing effect of light on the control protoplasts during the first days of culture (Enzmann-Becker, 1973). To mimick the growth-retardating effect of the UV-treatment, untreated protoplast suspensions were cultured at 6–7° C for 5 days in one experiment.

After one month, the cell colonies which had developed from the surviving protoplasts were scratched off the bottom of the Petri dishes and mixed with equal volumes of melted T_0 medium containing 0.6% agar and various concentrations of L-valine. After 2–5 months, microcalli which had survived this selective procedure were transferred to a regeneration medium, either R2 (medium T_0, but with $2.3 \cdot 10^{-6}$ M indoleacetic and $1.2 \cdot 10^{-6}$M kinetin for phytohormones and no mannitol) or R4 (Bourgin and Missonier,

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1978). Buds obtained on these media were rooted on medium B (Bourgin and Missonier, 1978) and the plantlets which had developed were vegetatively propagated by the in vitro cutting procedure mentionned above (Bourgin and Missonier, 1973) and then transferred to the greenhouse. Valine-resistance of the regenerated plants was assessed by culturing shoot pieces (1 cm long) possessing one axillary bud on medium B containing 2 mM L-valine.

Root tips of regenerated plants were analyzed cytologically (Bourgin and Missonier, 1978). These plants were selfed, when possible, and crossed to the wild-type plants, originating either from seed or from a diploidized plantlet obtained through leaf culture in vitro of the haploid clone (Kasperbauer and Collins, 1972). Progeny testing for valine-resistance was carried out by germinating 50 seeds from each cross on medium B containing 2 mM L-valine. This concentration permited the germination, but inhibited the development of non resistant phenotypes.

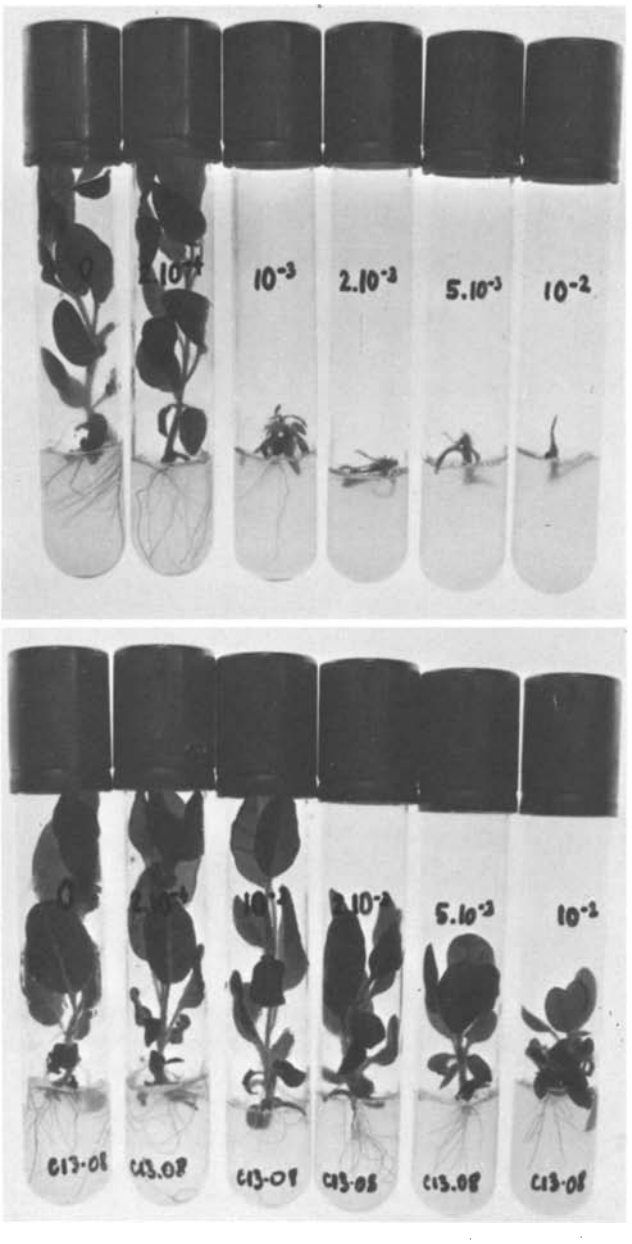

Fig. 1. Growth of shoot explants with one axillary bud from valine-resistant plant Val^r–1 (lower row) and from original haploid wild type plant (upper row) as a function of the concentration of L-valine in the culture medium B (from left to right: 0, 0.2, 1, 2, 5, 10 mM). Photographs were taken after a 7-week growth period

Results

Preliminary experimentation had demonstrated that concentrations of 0.5 to 1 mM valine temporarily inhibited growth of three-week old cell colonies derived from protoplasts, but eventually numerous colonies appeared. However, among 7 plants regenerated from such colonies none was found to be resistant to valine. Thus, higher concentrations of valine were used in subsequent selection experiments (2, 4.3 and 8.6 mM). No colony was recovered from plates with the highest concentration tested (8.6 mM). On plates containing 4.3 mM valine, 9 colonies were recovered out of $5 \cdot 10^5$ colonies that developped from the irradiated protoplasts, and none among 10^6 colonies from the control population.

In another experiment, using 2 mM valine, 6 colonies were recovered among $6 \cdot 10^5$ colonies that developed from the irradiated protoplasts and 5 among $1.2 \cdot 10^6$ colonies from the control population, which had been exposed to low temperature.

Among the 9 colonies recovered in the experiment using 4.3 mM valine, 2 did not survive transfer to regeneration medium R2 without valine, 3 developed further but did not form buds, and 4 gave rise to buds and then to plantlets. Shoot explants from only one of the regenerated plantlets developed on a medium containing 2 mM valine and this line was thus identified as valine-resistant (Val^r–1). A further test with a range of valine concentrations (Fig. 1) indicated that a 50% growth reduction of the explants from this line was observed at 10 mM valine, which is 10 times greater than the concentration required for wild type explants (Fig. 2).

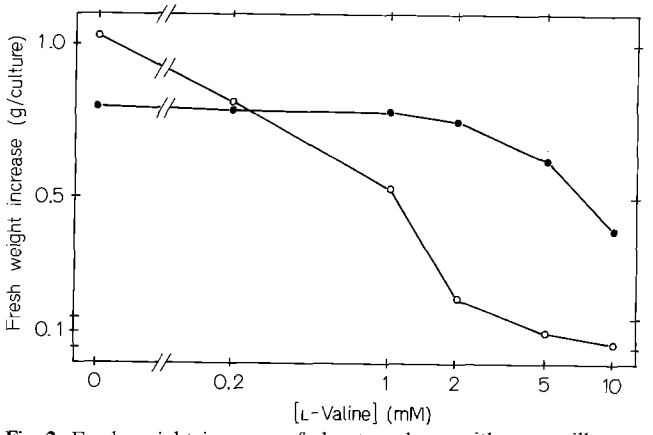

Fig. 2. Fresh weight increase of shoot explants with one axillary bud from haploid original wild type (○) and from valine-resistant Val^r–1 (●) tobacco plants as a function of the concentration of L-valine in the culture medium B. Each value is an average of six individual cultures scored after a 7-week growth period

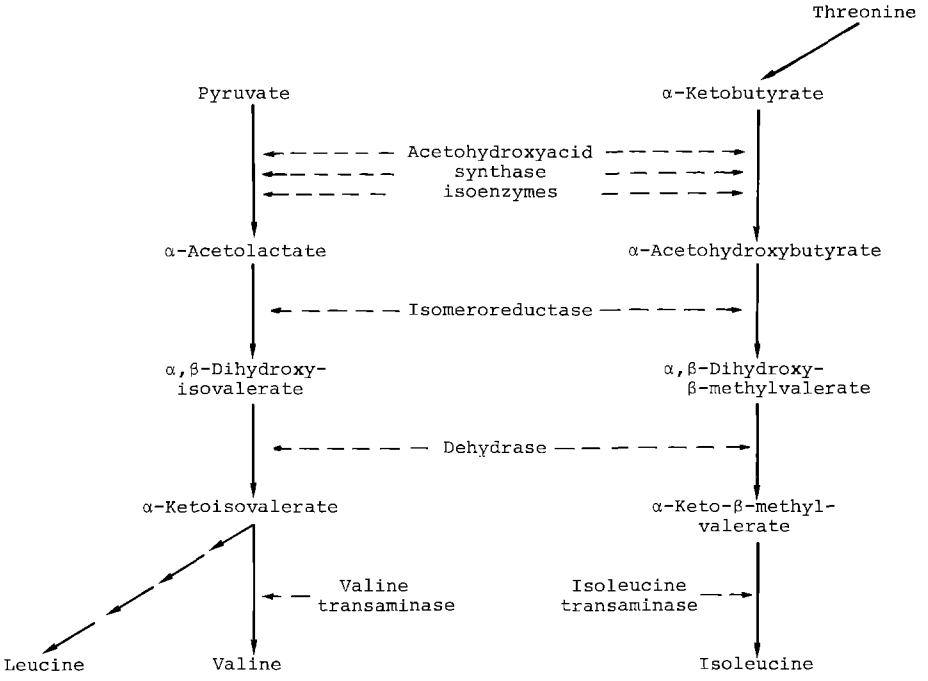

Fig. 3. The biosynthetic pathway for isoleucine, leucine and valine in *Escherichia coli* K-12 (adapted from De Felice et al., 1977)

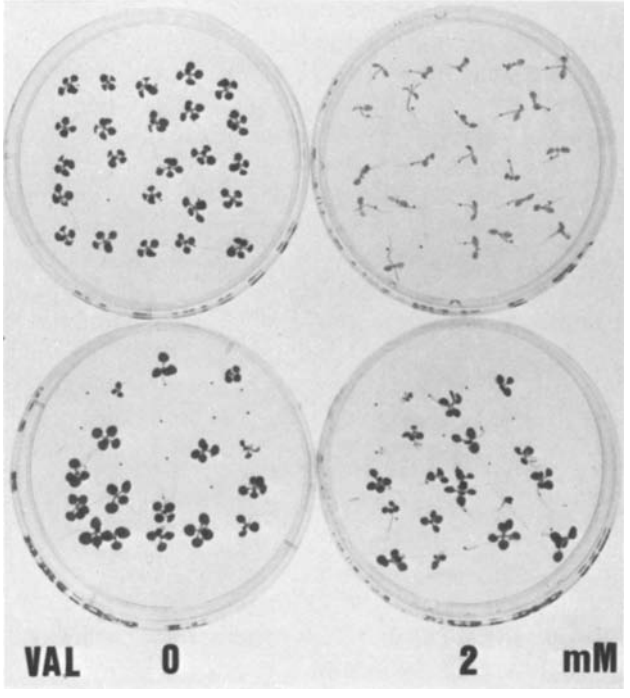

Fig. 4. Transmission of valine-resistance in F_1 progeny. Seeds from resistant plant Val^r-1 pollinated with pollen from wild type plant (lower row) and from selfed wild type plant (upper row) were germinated aseptically on medium B containing 0 or 2 mM valine. Photographs were taken 15 days later

Table 1. Transmission of valine-resistance in F_2 progenies from Val^r-1 plant. The F_1 plant used was obtained by pollinating a Val^r-1 plant with wild type (WT) pollen

Origin of the seedlings	Non resistant	Resistant
F_1 selfed	16	34
$F_1 \times WT$	23	21
$WT \times F_1$	22	27

In the experiment using 2 mM valine only 3 colonies from the irradiated population and 3 from the control survived the transfer to regeneration medium R4 containing 1 mM valine. Buds regenerated from all colonies, except one from the control fraction, survived the transfer to rooting medium B containing 1 mM valine and were therefore considered to be probably valine-resistant. Thus far, however, adult plants have been obtained from only one line (Val^r–2), originating from the irradiated fraction.

The morphology of the resistant plants appeared normal. However, while Val^r–2 plants were found to be diploid ($2n=48$) and self-fertile, Val^r–1 plants were male-sterile and displayed chromosome number ranging from 41 to 49. Val^r–2 seedlings obtained from selfing were all resistant to 2 mM valine, while those obtained from reciprocal backcrosses with wild-type plants were resistant only to 1 mM valine. Val^r-1 seedlings obtained from a backcross with a wild-type plant which was used as the pollen donor were all resistant to 2 mM valine (Fig. 4). Tests carried out with a range of valine concentrations on fragments

from two of these F_1 plantlets have shown that they were as resistant as the parental plant Val^r–1 (results not presented). One diploid plant from this F_1 generation was selfed and backcrossed to wild-type. Results of the test of these F_2 progenies are presented in Table 1.

Discussion

The valine-resistance traits (Val^r–1 and Val^r–2) are transmissible through sexual crosses. In each case, inheritance of valine-resistance might be explained by a single allele, dominant in the case of Val^r–1, and partially dominant in the case of Val^r–2. Thus, these variant phenotypes can be considered as resulting from mutations. The isolation of these mutants with this selection procedure reaffirms the uselfulness of protoplast suspensions as a tool in plant cell genetics (Carlson, 1973; Maliga et al., 1976; Aviv and Galun, 1977).

The two valine-resistant plants isolated thus far originated from the irradiated protoplast population. Since ultraviolet irradiation killed a fraction of the treated protoplasts and delayed division of the surviving ones, colony suspensions obtained from the irradiated population were not strictly comparable to those developed from the control population. Consequently the conditions used for selection could have been better suited for both the irradiated suspension and the cold-treated control. At least for this reason, the results reported here can not be considered as conclusive evidence for the mutagenic efficiency of ultraviolet irradiation on plant protoplasts. Mutagenic effect of UV-irradiation on pollen grains has been well documented (e.g. Pfahler and Linskens, 1977) and limited evidence of this effect on plant cells cultured in vitro has also been reported (Eriksson, 1967; Widholm, 1974b). Thus the use of UV-irradiation to mutagenize protoplasts could gain wider acceptance.

The selective procedure utilized in this study was similar to that employed by Carlson (1973) for isolating methionine sulfoximine-resistant tobacco lines, except for some minor modifications, the most notable being that protoplasts in this study were cultured in liquid instead of agar-solidified medium. However, this would appear to be of little consequence since colonies which developed from protoplasts in liquid medium did not appear to dissociate before or during the selective plating; the resistant colonies were thus likely to originate from one mutational event. Further experiments would be required to define other parameters known to be of importance in the in vitro isolation of mutants, such as i) the optimal time for applying the mutagenic treatment, ii) the optimal delay for the selective plating, and iii) the possible need for dissociating the colonies prior to selective plating (e.g.: Fox, 1975). Since I was primarily interested in selecting genetic alterations transmissible to whole plants, the valine-resistance of presumptive mutant cell lines was tested on standardized explants from regenerated plantlets. Obviously, valine-resistant cell lines which had lost their capacities for bud regeneration under the conditions tested would have escaped such a procedure of retesting. Regeneration of plants sensitive to valine from calli recovered on the selective medium confirms previous observations reported with other mutant cell lines selected in vitro (Carlson, 1973; Sung, 1976) and underlines the necessity of retesting presumptive mutant lines isolated under such conditions. Several phenomena could account for this feature.

a) If colonies derived from mutated protoplasts are chimeric and if wild type cells in association with resistant ones are able to survive the selection pressure, wild type plants could conceivably be recovered. Apparently this was not the case with Val^r–2 colony since all 9 buds which were regenerated from this colony were found to be valine-resistant.

b) Certain genetic or epigenetic alterations conferring valine-resistance could be expressed at the colony or callus stage but not in the derived plantlet. Auxin- and cytokinin-habituation (Lutz, 1971; Binns and Meins, 1973) or the transient cycloheximide-resistance observed by Maliga et al. (1976) in shoot-forming calli are likely examples of such epigenetic alteration. To my knowledge no confirmed plant biochemical mutant with such a phenotype has yet been described.

c) A more trivial possibility is that the selective conditions employed were not stringent enough to prevent the survival of colonies either of the wild type phenotype or of modified phenotypes whose growth was favored in adverse conditions.

Various problems were encountered in the process of plant regeneration through bud neoformation from the selected colonies.

a) Some calli were unable to form buds on the regeneration medium which was used. This loss of regenerative capacity is common in tissues or cell suspensions which have been submitted to repeated subculture (e.g.: Smith and Street, 1974; Widholm, 1974b). The genetic alterations likely responsible for this loss could have taken place during the development of callus in the selective medium. Ultraviolet irradiation of isolated cells has also been shown to partially suppress their regenerative capacity (Eapen, 1976). Finally, one cannot exclude the possibility that certain valine-resistance mutations are incompatible with the regeneration process.

b) Diploid and near-diploid plants were regenera-

ted from presumed haploid protoplasts. In vitro culture conditions can account for endopolyploidization and aneuploidization (Sacristán and Melchers, 1969; Takebe et al., 1971; Ogura, 1976), but it could also have been possible that regenerated plants originated from spontaneously fused protoplasts (Withers and Cocking, 1972). In this eventuality, however, it is likely that only one of the two chromosome complements would have carried the allele responsible for the valine-resistance phenotype. Since the progeny testing of valine-resistant plants did not show heterozygosity for this trait it must be assumed that diploidization occured after the mutation.

The mechanism of valine-resistance is not yet known. Different biochemical alterations could account for this trait.

a) Uptake of exogenous valine may have been reduced in the resistant cells. Decreased uptake of tryptophan and of its analogs was considered by Widholm (1974a) to be probably responsible for 5-methyltryptophan-resistance in one carrot cell line.

b) As in microorganisms (Umbarger, 1969; De Felice et al., 1977), the last steps of the biosynthetic pathways leading to valine and to isoleucine are probably catalyzed in higher plants by the same enzymes (Bryan, 1976) (Fig. 3). In bacteria, the regulation of these pathways is insured, in part, by control of the first enzyme common to both pathways, acetohydroxyacid synthase; this control may involve feedback inhibition of enzyme activity and repression of enzyme synthesis (Umbarger, 1969; De Felice et al., 1977). In higher plants, whereas there is no evidence of enzyme repression, cooperative feedback regulation of acetohydroxyacid synthase by leucine and valine has been reported for all species tested (Miflin and Cave, 1972) with the exception of *Phaseolus radiatus* (Satyanarayana and Radakrishnan, 1963). Although more inhibitory when supplied together, leucine and valine are significantly inhibitory on their own (Miflin and Cave, 1972). Thus growth inhibition of tobacco cells by exogenously supplied valine could be due to inhibition of the acetohydroxyacid synthase by excess valine, leading to starvation of the cells for isoleucine. An altered acetohydroxyacid synthase more resistant to feedback inhibition by valine could confer resistance to valine-resistant cells. Such a phenomenon has been observed by Widholm (1972a; b) with anthranilate synthase from 5-methyltryptophan-resistant cell lines of tobacco and carrot.

c) Valine also belongs to the group of aminoacids known to inhibit the growth of plant cells when they are cultivated on nitrate as the sole source of nitrogen (Filner, 1966; Behrend and Mateles, 1975). This effect may have prevailed in the selective conditions and thus, the valine-resistance observed here would be comparable to the threonine-resistance reported with the tobacco cell line isolated by Heimer and Filner (1970).

Nutritional and biochemical experiments should elucidate the nature of the alteration. However, irrespective of the mechanism involved, this report is the first to my knowledge to describe the successful selection of amino acid-resistant plants. Such mutants could be of great value in studying the regulation of amino acid biosynthesis (Miflin and Lea, 1977; Green and Phillips, 1974) or the amino acid-sensitive steps in primary nitrogen metabolism (Behrend and Mateles, 1975).

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References

Acedo, G.N., Redéi, G.P.: Regulation of amino acid metabolism in *Arabidopsis*. Arabidopsis Inf. Serv. **13**, 120–124 (1976)

Aviv, D., Galun, E.: Isolation of tobacco protoplasts in the presence of isopropyl N-phenylcarbamate and their culture and regeneration into plants. Z. Pflanzenphysiol. **83**, 267–273 (1977)

Behrend, J., Mateles, R.I.: Nitrogen metabolism in plant cell suspension cultures. I. Effect of amino acids on growth. Plant Physiol. **56**, 584–589 (1975)

Binns, A., Meins, F.: Habituation of tobacco pith cells for factors promoting cell division is heritable and potentially reversible. Proc. nat. Acad. Sci. (Wash.) **70**, 2660–2662 (1973)

Bourgin, J.P.: Valine-induced inhibition of growth of haploid tobacco protoplasts and its reversal by isoleucine. Z. Naturforsch. **31c**, 337–338 (1976)

Bourgin, J.P., Missonier, C.: Vegetative propagation and cold preservation of haploid plants of *Nicotiana tabacum* and *Nicotiana paniculata*. Haploid Inf. Serv. **8**,7 (1973)

Bourgin, J.P., Missonier, C.: Culture de protoplastes de mésophylle de *Nicotiana alata* Link et Otto haploïde. Z. Pflanzenphysiol. **87**, 55–64 (1978)

Bourgin, J.P., Missonier, C., Chupeau, Y.: Culture de protoplastes de mésophylle de *Nicotiana sylvestris* Spegazzini et Comes haploïde et diploïde. C.R. Acad. Sci. (Paris), Ser. D **282**, 1853–1856 (1976)

Bryan, J.K.: Amino acid biosynthesis and its regulation. In: Plant biochemistry (J. Bonner and J.E. Varner, eds.), pp. 525–560. New York-London: Academic Press 1976

Carlson, P.S.: The use of protoplasts for genetic research. Proc. nat. Acad. Sci. (Wash.) **70**, 598–602 (1973)

Chupeau, Y., Bourgin, J.P., Missonier, C., Dorion, N., Morel, G.: Préparation et culture de protoplastes de divers *Nicotiana*. C.R. Acad. Sci. (Paris) **278**, 1565–1568 (1974)

De Felice, M., Squires, C., Levinthal, M., Guardiola, J., Lamberti, A., Iaccarino, M.: Growth inhibition of *Escherichia coli* K.12 by L-valine: a consequence of a regulatory pattern. Molec. gen. Genet. **156**, 1–7 (1977)

Eapen, S.: Effect of gamma- and ultraviolet-irradiation on survival and totipotency of haploid tobacco cells in culture. Protoplasma **89**, 149–155 (1976)

Enzmann-Becker, G.: Plating efficiency of protoplasts of tobacco in different light conditions. Z. Naturforsch. **28c**, 470–471 (1973)

Eriksson, T.: Effects of ultraviolet and X-ray radiation on in vitro cultivated cells of *Haplopappus gracilis*. Physiol. Plant. **20**, 507–518 (1967)

Filner, P.: Regulation of nitrate reductase in cultured tobacco cells. Biochim. biophys. Acta (Amst.) **118**, 299–310 (1966)

Fox, M.: Factors affecting the quantitation of dose-response curves for mutation induction in V 79 chinese hamster cells after exposure to chemical and physical mutagens. Mutation Res. **29**, 449–466 (1975)

Green, C.E., Phillips, R.L.: Potential selection system for mutants with increased lysine, threonine, and methionine in cereal crops. Crop Sci. **14**, 827–830 (1974)

Heimer, Y.M., Filner, P.: Regulation of the nitrate assimilation pathway of cultured tobacco cells. II. Properties of a variant cell line. Biochim. biophys. Acta (Amst.) **215**, 152–165 (1970)

Henke, R.R., Wilson, K.G., Mc Clure, J.W., Treick, R.W.: Lysine-methionine-threonine interactions in growth and development of *Mimulus cardinalis* seedlings. Planta (Berl.) **116**, 333–345 (1974)

Kasperbauer, M.J., Collins, G.B.: Reconstitution of diploids from leaf tissue of anther-derived haploids in tobacco. Crop Sci. **12**, 98–101 (1972)

Lutz, A.: Aptitudes morphogénétiques des cultures de tissus d'origine unicellulaire. In: Les cultures de tissus de plantes, pp. 163–168. Paris: Coll. Inter. C.N.R.S. No. 193, Editions du Centre Nat. Rech. Scient. 1971

Maliga, P.: Isolation of biochemical mutants in tissue cultures of flowering plants. Arabidopsis Inf. Serv. **13**, 193–199 (1976a)

Maliga, P.: Isolation of mutants of cultured plant cells. In: Cell genetics in higher plants (D. Dudits, G.L. Farkas, P. Maliga, eds.), pp. 59–77. Budapest: Publishing House of the Hungarian Academy of Sciences 1976b

Maliga, P., Lázár, G., Sváb, Z., Nagy, F.: Transient cycloheximide resistance in a tobacco cell line. Molec. gen. Genet. **149**, 267–271 (1976)

Miflin, B.J.: The inhibitory effects of various amino acids on the growth of barley seedlings. J. exp. Bot. **20**, 810–819 (1969)

Miflin, B.J., Cave, P.R.: The control of leucine, isoleucine, and valine biosynthesis in a range of higher plants. J. exp. Bot. **23**, 511–516 (1972)

Miflin, B.J., Lea, P.J.: Amino acid metabolism. Ann. Rev. Plant Physiol. **28**, 299–329 (1977)

Ogura, H.: The cytological chimeras in original regenerates from tobacco tissue cultures and in their offsprings. Jap. J. Genet. **51**, 161–174 (1976)

Palmer, J.E., Widholm, J.M.: Characterization of carrot and tobacco cell cultures resistant to p-fluorophenylalanine. Plant Physiol. **56**, 232–238 (1975)

Pfahler, P.L., Linskens, H.F.: Ultraviolet irradiation of maize (*Zea mays* L.) pollen grains. II. Pollen genotype effects on plant characteristics. Theoret. appl. Genet. **50**, 17–21 (1977)

Sacristán, M.D., Melchers, G.: The caryological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Molec. gen. Genet. **105**, 317–333 (1969)

Sandstedt, R., Skoog, F.: Effects of amino acid components of yeast extract on the growth of tobacco tissue *in vitro*. Physiol. Plant. **13**, 250–256 (1960)

Satyanarayana, T., Radhakrishnan, A.N.: Biosynthesis of valine and isoleucine in plants. I. Formation of α-acetolactate in *Phaseolus radiatus*. Biochim. biophys. Acta (Amst.) **77**, 121–132 (1963)

Smith, S.M., Street, H.E.: The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. Ann. Bot. **38**, 223–241 (1974)

Street, H.E.: The nutrition and metabolism of plant tissue and organ cultures. In: Cells and tissues in culture. Methods, biology and physiology, Vol. 3 (E.N. Willmer, ed.), pp. 533–629. London-New York: Academic Press 1966

Sung, Z.R.: Mutagenesis of cultured plant cells. Genetics **84**, 51–57 (1976)

Takebe, I., Labib, G., Melchers, G.: Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften **58**, 318–320 (1971)

Umbarger, H.E.: Regulation of the biosynthesis of the branched-chain amino acids. Curr. Top. Cell Regul. **1**, 57–76 (1969)

Widholm, J.M.: Cultured *Nicotiana tabacum* cells with an altered anthranilate synthetase which is less sensitive to feedback inhibition. Biochim. biophys. Acta (Amst.) **261**, 52–58 (1972a)

Widholm, J.M.: Anthranilate synthetase from 5-methyltryptophan-susceptible and -resistant cultured *Daucus carota* cells. Biochim. biophys. Acta (Amst.) **279**, 48–57 (1972b)

Widholm, J.M.: Cultured carrot cell mutants: 5-methyltryptophan-resistance trait carried from cell to plant and back. Plant Sci. Lett. **3**, 323–330 (1974a)

Widholm, J.M.: Selection and characteristics of biochemical mutants of cultured plant cells. In: Tissue culture and plant science (H.E. Street, ed.), pp. 287–299. New York and London: Academic Press 1974b

Widholm, J.M.: Selection and characterization of cultured carrot and tobacco cells resistant to lysine, methionine, and proline analogs. Canad. J. Bot. **54**, 1523–1529 (1976)

Widholm, J.M.: Selection and characterization of biochemical mutants. In: Plant tissue culture and its bio-technological applications (W. Barz, E. Reinhard, M.H. Zenk, eds.)), pp. 112–122. Berlin-Heidelberg-New York: Springer 1977

Withers, L.A., Cocking, E.C.: Fine structural studies on spontaneous and induced fusion of higher plant protoplasts. J. Cell Sci. **11**, 59–75 (1972)

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