GENETIC CONTROL OF *IN VITRO* PLANT REGENERATION AND EFFECTS OF KINETIN ON CALLUS CHARACTERS IN ALFALFA (*MEDICAGO SATIVA* L.)

by

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INDEX WORDS

Tissue culture, callus, segregation ratio, complementary genes.

SUMMARY

The genetic control of plant regeneration from callus culture was studied in tetraploid alfalfa (*Medicago sativa*, L.). Seven cultivars (total 72 plants) were screened for regenerability. Ladak had the best regeneration response, in which 42% of the plants regenerated. Four regenerable plants and three nonregenerable plants were used to form 10 F1 hybrids and three S1 populations. Segregation ratios in the populations suggested that regenerability of alfalfa via petiole culture was under the control of two complementary genes, Rn1 and Rn2. The presence of both dominant genes was necessary for a plant to regenerate in a two-step culture system. The data also indicated that gene dosage influenced regeneration efficiency. Significant reciprocal effects demonstrated that the interaction between callus initiation medium and callus regenerability

was affected by cytoplasmic factor(s).

INTRODUCTION

Successful *in vitro* regeneration of many plant species has resulted from proper growth medium and environmental conditions (MURASHIGE, 1974) as well as genetic control (BROWN & ATANASSOV, 1985; NESTICKY et al., 1983; OELCK & SCHIEDER, 1983; REISCH & BINGHAM, 1980). The effects of genetic background on plant tissue culture have been increasingly noted. TABATA and MOTOYOSHI (1965) first reported major gene and maternal effects in callus formation from maize (*Zea mays* L.) endosperm. SUN & ULLSTRUP (1971) also found that proliferation of endosperm callus in maize was controlled by two genetic factors, with the expression of a maternal effect. IZHAR and POWER (1977) suggested that only a few genes were involved in genotype-specific hormone requirements for protoplast growth in petunia (*Petunia* sp.) and different stages of protoplast development might be controlled by different genes. MA et al. (1987) suggested that two complementary genes were involved in regeneration from cultured immature embryos of sorghum (*Sorghum vulgare*). HLASNIKOVA (1977) studied the genetic aspects of *in vitro* androgenesis in tobacco (*Nicotiana* sp.) species and found that genetic interactions at the level of species, lines, and hybrids played an important part in promoting androgenetic

efficiency. BUIATTI et al. (1974) determined that callus growth and bud formation in wild cabbage (Brassica oleracea L.) was controlled primarily by additive gene effects. Dependence of regeneration on genotype in tissue culture has been observed in legumes such as Arachis, Glycine, Melilotus, Vicia (PHILLIPS, 1983), Trifolium (CAMPBELL & TOMES, 1984), Cajanus (KUMAR et al., 1983), Coronilla (MARIOTTI & ARCIONI, 1983), Phaseolus, Stylosanthes (MEIJER, 1982), Lotus (KEYES et al., 1980) and Medicago (ATANASSOV & BROWN, 1984; BINGHAM et al., 1975). In alfalfa, genotypic variation in embryogenesis appears to be a widespread phenomenon. BROWN & ATANASSOV (1985) found that embryogenesis response in cell suspensions and callus cultures derived from cotyledon explants was strongly genotype dependent. The induction of somatic embryogenesis varied among cultivars (BINGHAM et al., 1975; BROWN & ATANASSOV, 1985) and genotypes of a cultivar (KAO, 1981; MITTEN et al., 1984; PHILLIPS, 1983). Even though the frequency of regenerating genotypes within a cultivar was high, much variability existed in the efficiency of regeneration (MITTEN et al., 1984). This was attributed to the intervarietal and intravarietal heterogeneity in alfalfa which is an open-pollinated species.

Plant regeneration from callus was highly heritable in alfalfa. Regeneration increased from about 10% in standard

alfalfa cultivars to 67% in two cycles of recurrent selection (BINGHAM et al., 1975). REISCH & BINGHAM (1980) found that in diploid alfalfa, bud differentiation from callus was controlled by two dominant genes, and both must be present in order to obtain more than 75% regeneration. They designated the genes as Rn1 and Rn2.

In genetic studies of tissue culture, a cytoplasm effect was noted by some researchers. NESTICKY et al. (1983) noted significant reciprocal effects in the basic analysis of combining ability and high values of reciprocal effects in corn tissue culture. They suggested that callus growth in vitro was controlled by two genetic systems, one located in the nucleus and another in the cytoplasm. Proliferation of endosperm callus of corn also was considered to be controlled by two genetic factors, with the expression of a maternal effect (SUN & ULLSTRUP, 1971; TABATA & MOTOYOSHI, 1965).

These genetic studies of different species in tissue culture indicated that several major traits are probably under qualitative genetic control, and cytoplasm may make a contribution. Our study was designed to assess the qualitative genetic control of in vitro regeneration of tetraploid (2n=4x=32) alfalfa. The effect of cytoplasm on the interaction between callus initiation medium and callus regenerability also was studied by comparing the responses of the F1 progenies of two sets of reciprocal crosses, in

which two parents of each set of crosses had different responses to two initiation media.

MATERIALS AND METHODS

A total of 72 plants from seven cultivars: 'Ladak', 'Lahontan', 'Grimm', 'DuPuits', 'Buffalo', 'Anik', and 'African', were screened for regenerability. Each plant was numbered, e.g., Ladak-1, Lahanton-17 etc. Three regenerable plants and one nonregenerable plant from Ladak and one regenerable and two nonregenerable plants from Lahontan were selected as parents to produce 10 F1 populations and three S1 populations (Table 1). Populations I and II are F1 populations derived from crosses between two nonregenerable parents. Populations III to VII are F1 populations from crosses between one regenerable parent and one nonregenerable parent. Populations VIII to X are F1 populations between two regenerable parents. Populations XI and XII are S1 progenies of regenerable plants. Population XIII is S1 progeny of a nonregenerable plant. All the crosses were made in a greenhouse by hand pollination without emasculation. Parents and their progenies were grown in the same greenhouse.

A two-step sequence was used in this study. Medium 7951 (LIANG, 1982) containing 2 mg/l 2,4-D and 0.5 mg/l kinetin solidified with Difco agar was used for callus initiation.

A hormone free medium, SHAP, which is a modified SCHENK and HILDEBRANDT (1972) medium without hormone and with an additon of 50 um proline and 30 um alanine, was used for regeneration. Both media were adjusted to pH 5.9-6.0. Petioles of the second or third leaves from the stem apex were used as explants for callus initiation. Petioles were cut into pieces (about 0.5 cm long) which were sterilized in 75% ethanol for 15 seconds and then in 50% commercial bleach for 5 minutes followed by three washes in sterile double distilled water. Sterilized petiole segments, usually 11 to 13 pieces, were cultured on about 25 ml 7951 medium in each Petri dishes (100x15 mm). Petri dishes were sealed with Parafilm. All the cultures were maintained at 25 $\pm$ 1 C in a dark incubator. After one month, part of each induced callus tissue was transferred to the regeneration medium (SHAP) and maintained under the same conditions as above. Generally, calluses derived from regenerable plants started showing embryogeneses 1 week following transfer to SHAP. These dishes of SHAP were moved to an incubator with 12-hour photoperiod at 25 $\pm$ 1 C for plantlet development. Regeneration was scored 1 month later. The regeneration efficiency of plants was calculated as percentage of calluses regenerating plantlets.

Calluses were measured and divided into four classes according to width in mm:

1 = greater than or equal to 5.0, 2 = 4.0 to 4.9, 3 =3.0

to 3.9, 4 = less than 3.0.

Analyses were made from results of two replications. A plant that showed regenerability in either of two replications was counted as a regenerable plant, i.e. a plant capable of regeneration from patiole-derived callus. Chi-square tests for goodness of fit were used to analyze the segregation ratios. The expected segregation ratio of each population was determined from its proposed parental genotype. In order to document regenerability of the parents, they were recultured with their progenies.

For studying the effect of cytoplasm on the interaction between callus initiation medium and callus regenerability, four F1 populations from two sets of reciprocal crosses [(Ladak-1 x Ladak-28, Ladak-28 x Ladak-1) and (Ladak-42 x Ladak-1, Ladak-1 x Ladak-42)] were used to analyze the regeneration responses to two initiation media, 7951 and B2-k. B2-k is a modified B2 medium (SAUNDERS & BINGHAM, 1975) with 2 mg/l of 2,4-D but no kinetin or NAA. In a preliminary study, Ladak-1 was regenerable on SHAP medium with either 7951 or B2-k as the callus initiation medium, but Ladak-28 and Ladak-42 were regenerable on SHAP only through 7951.

RESULTS AND DISCUSSION

Among seven cultivars, Ladak had the best plant regeneration response (42%) in this culture system. Only

17, 11, and 9% of plants regenerated from DuPuits, Buffalo, and Lahontan, respectively. No regenerable plants were noted from Grimm, Anik, and African. These results corroborate those of BROWN & ATANASSOV (1985). When screening 76 alfalfa cultivars (M. sativa L., M. falcata L. and M. varia Martyn), they found that high regenerating cultivars contained a strong genetic contribution from two land-race germplasm sources (M. falcata and Ladak) in their ancestry. Three of four regenerable parents we used to form F1 and S1 populations for the genetic study of plant regeneration were selected from Ladak.

The numbers of regenerable plants and nonregenerable plants in the F1 and S1 populations are summarized (Table 1). Since each of Populations VIII, IX, and X was developed from a cross between two regenerable plants, segregations of F1 plants (Table 1) indicated that callus regenerability was controlled by dominant gene(s). In two F1 populations (I and II) derived from crosses of two nonregenerable parents, some F1 plants regenerated. This suggested that more than one gene was necessary for regeneration. We thus considered a model of two complementary genes. In this model, regenerability is dependent on two dominant genes at two loci. Since REISCH and BINGHAM (1980) named the genes as Rn1 and Rn2 in diploid alfalfa, we proposed that the genotype of a regenerable plant is Rn1---Rn2--- and the

genotype of a nonregenerable plant is Rn1---rn2rn2rn2rn2, rn1rn1rn1rn1Rn2---or rn1rn1rn1rn1rn2rn2rn2rn2.

REISCH & BINGHAM (1980), based on a study of diploid alfalfa, suggested that bud differentiation from callus was controlled by two dominant genes. They examined the efficiency of regeneration and suggested that a pair of dominant genes at two loci (Rn1 and Rn2) were necessary for the expression of regeneration efficiency of more than 75%. A plant with either Rn1, or Rn2 was regenerable although at low afficiency. In contrast to their suggestion, we considered that regeneration was the function of the complementation of these two genes. Genotype Rn1---Rn2--- was necessary for a plant to regenerate.

Based on our hypothesis, the most reasonable genotype was determined for each parent (Table 2), and tested with the segregation ratio in each population. The results of Chi-square tests demonstrated that this model was acceptable in all populations but Population IV (Table 3). In most of the populations (I, III, V, VI, XI, XII and XIII), the segregation ratios corresponded well to the expected ones. No regenerable plants appeared in Population XIII, the S1 population of a nonregenerable plant (Lahontan-1). This plant was one of the two nonregenerable parents producing Population II (Table 1). Because some regenerable plants appeared in Population II, Lahantan-1 could not be a homozygous recessive plant

(rn1rn1rn1rn1rn2rn2rn2rn2).

The parents selected to obtain hybrids and S1 progenies all callused readily on the 7951 medium. The progenies varied little in callus initiation, growth, and morphology. Most of the calluses were in the two top groups (Table 4). Each culture had sufficient callus for transfer to the SHAP medium to induce regeneration. This indicated that callus production and callus regenerability of a plant were controlled by different genetic factors. The results from Population XIII confirmed this assumption, since no plant in this population regenerated but callus initiation, growth and morphology were similar to those in populations that did regenerated. BROWN & ATANASSOV (1985) also concluded that good callus production was not a prerequisite for a high regeneration response. Callus formation, however, was required for plant regeneration.

Among regenerable plants, differences in the efficiency of callus regeneration (percentage of calluses from each plant regenerating plants) were noted. Three possible explanations may be used to describe variations in regeneration efficiency among regenerators: 1) In addition to the major genes for regenerability, there may be modifiers influencing the efficiency. It is difficult, however, to confirm it. 2) There may be separate genetic systems controlling regeneration efficiency. The difference

between our results and those of REISCH & BINGHAM (1980) suggests that a different genetic system may exist. 3) The difference in regeneration efficiency may be due to gene dosage effect. That is, in the presence of Rn1 and Rn2, the number of dominant alleles at both loci determines the efficiency. In our study, two observations supported this possibility: 1) Among regenerable parental plants, Ladak-12 had the highest regeneration efficiency. According to the segregation ratios of those populations in which Ladak-12 was used as a parent, the proposed genotype for Ladak-12 was Rn1Rn1rn1rn1Rn2Rn2rn2rn2. There are one or two more dominant alleles in this genotype than in those proposed for other regenerable parents (Table 3). 2) In a comparison of sib-lines, Population II with Population III, Population VIII and IX with Population X (Table 5), more F1 plants had high regeneration efficiency when Ladak-12 was used as a parent. Results were consistent with the expected segregation ratios and suggested that regeneration efficiency was affected by gene dosage.

Cytoplasm apparently contributes to the interaction between callus induction medium and callus regenerability. When Ladak-1 was the female parent in reciprocal crosses, more F1 plants regenerated on SHAP medium through B2-k than when Ladak-28 or Ladak-42 was the female (Table 6). Among the F1 plants of Ladak-28 x Ladak-1, only 1 of 24 plants regenerated on SHAP through B2-k. In contrast, 10 of 22 F1

plants of the reciprocal cross regenerated. Since Ladak-1 regenerated on SHAP with either 7951 or B2-k as the induction medium, and Ladak-28 and Ladak-42 regenerated on SHAP only through 7951, a cytoplasm effect on the interaction between callus induction medium and callus regenerability is suggested. Thus, the direction of crossing might ultimately influence regenerability of the progeny.

Since alfalfa is an open-pollinated crop and plants of a cultivar are highly heterogeneous and heterozygous, inbreeding depression of plant growth and some other traits is serious. Inbreeding reduced callus production (KEYES & BINGHAM, 1979), and bud and plant formation in diploid alfalfa (REISCH & BINGHAM, 1980). In our study, segregation ratios in Populations XI and XII, which are S1 progenies of Ladak-1 and Ladak-12, respectively, were very close to the expected ratios. Therefore, callus regenerability was minimally affected by inbreeding.

Our finding that regeneration is a character controlled by a few dominant genes agrees with that of other studies on genetic control in tissue culture. It should be relatively easy to transfer the regenerability to other plants. It also is desirable to find a closely linked marker gene to facilitate the identification of regenerable plants. Transfer of such a linkage would be useful for mass

plant regeneration from callus cultures.

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Table 1. Segregation of regenerability in F1 and S1 populations.

Population & Generation		Parent(s)	No. Plants reg.	No. Plants nonreg.
I	F1	Ladak-6(NR)xLahontan-21(NR)	6	19
II	F1	Lahontan-1(NR)xLadak-6(NR)	2	27
III	F1	Ladak-6(NR)xLadak-12(R)	12	3
IV	F1	Ladak-1(R)xLadak-6(NR)	26	9
V	F1	Ladak-28(R)xLahontan-21(NR)	17	12
VI	F1	Ladak-6(NR)xLahontan-17(R)	3	6
VII	F1	Lahontan-21(NR)xLahontan-17(R)	2	9
VIII	F1	Ladak-28(R)xLadak-1(R)	14	10
IX	F1	Ladak-1(R)xLadak-28(R)	19	3
X	F1	Ladak-28(R)xLadak-12(R)	42	2
XI	S1	Ladak-1(R)	26	11
XII	S1	Ladak-12(R)	20	2
XIII	S1	Lahontan-1(NR)	0	24

1 NR = nonregenerable plant, R = regenerable plant

Table 2. Proposed genotypes for parental plants.

Parent	Proposed genotype
Ladak-1	Rn1Rn1rn1rn1Rn2rn2rn2rn2
Ladak-6	Rn1rn1rn1rn1rn2rn2rn2rn2
Ladak-12	Rn1Rn1rn1rn1Rn2Rn2rn2rn2
Ladak-28	Rn1Rn1rn1rn1Rn2rn2rn2rn2
Lahontan-1	rn1rn1rn1rn1Rn2rn2rn2rn2
Lahontan-17	Rn1rn1rn1rn1Rn2rn2rn2rn2
Lahontan-21	rn1rn1rn1rn1Rn2rn2rn2rn2

Table 3. Segregation ratios and goodness of fit to a complementary gene model for the F1 and S1 populations.

population	No. plants Observed Reg	Nonreg	Expected Reg	Nonreg	P
I	6	19	6.3	18.7	>0.90
II	2	27	7	22	0.05--0.025
III	12	3	11.5	3.5	0.90--0.75
IV	26	9	16	19	<0.005
V	17	12	18	11	0.75--0.50
VI	3	6	3.4	5.6	0.95--0.75
VII	2	9	4.1	6.9	0.25--0.10
VIII	14	10	17.5	6.5	0.25--0.10
IX	19	3	16	6	0.25--0.10
X	42	2	39.2	4.8	0.25--0.10
XI	26	11	27	10	0.75--0.50
XII	20	2	20.8	1.2	0.50--0.25
XIII	0	24	0	24	undefined

Table 4. Distributions of plants by callus size in each population.

Population	No. plants cultured	No. plants in callus groups 1--2	2--3	3--4
I	25	23	1	1
II	29	25	4	0
III	15	10	5	0
IV	35	30	5	0
V	29	27	2	0
VI	9	9	0	0
VII	11	11	0	0
VIII	24	21	2	1
IX	22	19	3	0
X	44	42	2	0
XI	37	28	7	2
XII	22	14	7	1
XIII	24	20	4	0

1 1 =>5.0 mm, 2 =4.0 to 4.9 mm, 3 =3.0 to 4.9 mm, 4 <3 mm
Plants were classified on the basis of two replications. Callus size differences between replications varied up to one class so each group includes two classes.

Table 5. Distributions of plants among regeneration efficiency group.

Population	Parent(s)	% of reg plants in each reg efficiency group*			
		A	B	C	D
I	Ladak-6xLahontan-21	17	0	17	66
II	Lahontan-1xLadak-6	0	0	0	100
III	Ladak-6xLadak-12	50	25	17	8
IV	Ladak-1xLadak-6	24	40	16	20
V	Ladak-28xLahontan-21	7	36	36	21
VI	Ladak-6xLahontan-17	--	34	33	33
VII	Lahontan-21xLahontan-17	50**	--	--	50
VIII	Ladak-28xLadak-1	27	36	10	27
IX	Ladak-1xLadak-28	50	17	8	25
X	Ladak-28xLadak-12	57	12	14	17
XI	Ladak-1	27	18	--	55
XII	Ladak-12	29	29	27	15
XIII	Lahontan-1	No regenerable plant			

* 100% > Group A > 75%

75% > Group B > 50%

50% > Group C > 25%

25% > Group D > 0

** Only two regenerable plants in this population, one is in Group A, another is in Group D.

Table 6. Reciprocal effects on plant regeneration from callus initiated on different induction media

Reciprocal crosses	No.plants cultured	No.plants regenerable on SHAP through B2-k & 7951	7951	B2-k	% plants reg through B2-k
Ladak-1 x Ladak-28	22	8	11	2	48
Ladak-28 x Ladak-1	24	1	13	0	7
Ladak-1 x Ladak-42	11	9	2	0	82
Ladak-42 x Ladak-1	45	16	24	0	40

PART II

THE EFFECTS OF KINETIN ON CALLUS CHARACTERS IN ALFALFA (*MEDICAGO SATIVA* L.)

THE EFFECTS OF KINETIN ON CALLUS CHARACTERS IN ALFALFA (*MEDICAGO SATIVA* L.)

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INDEX WORDS

Callus culture, initiation, growth, morphology, histology, regeneration, cytokinin autotrophy.

SUMMARY

Three callus initiation media, B2-k, B2, and 7951, were used to study the effects of kinetin on callus initiation, morphology, histology, and regenerability. The presence of kinetin in callus initiation media retarded callus initiation, but enhanced division and differentiation of callus cells. Calluses induced on kinetin-containing media (B2 and 7951) had many compact cell aggregations, which were considered meristematic regions that might differentiate to plantlets on a regeneration medium. Visually, these calluses were compact and had many nodular structures. In contrast, most calluses induced on a kinetin-free medium were composed of large, individual cells and had friable structures without nodules. After transfer to a hormone-free medium, calluses induced on kinetin-containing media regenerated more frequently than

those induced on a kinetin-free medium, but cytokinin (kinetin) autotrophism also occurred. Autotrophism was sexually transmissable and especially affected by the female parent.

INTRODUCTION

Three factors appear to control regeneration of plants in tissue culture: 1) genetic background of culture, 2) medium, and 3) environment. Kinetin, one of the commonly used cytokinins in plant tissue culture, had important effects on callus development and regeneration. Kinetin was not necessary for callus initiation, but enhanced proliferation and shoot formation in tissue culture of Solanum carolinense (REYNOLDS, 1986). SUN & LU (1984) indicated that, in rice anther culture, kinetin had no stimulating effect on callus initiation, retarded callus formation and growth in vitro, and facilitated callus differentiation into green seedlings. Embryoids and calluses were obtained on unsupplemented basal media in maize anther culture. However, induction frequencies were generally better with added growth regulators, notably kinetin, either alone or with 2,4-D, or TIBA (ZHENG et al., 1983). In alfalfa callus culture, in which a two-step procedure was usually necessary, the presence of kinetin in the first medium was not required for budding but its

presence increased bud formation from 123-160% (SAUNDERS & BINGHAM, 1975). WALKER et al. (1978) demonstrated that regeneration was under quantitative hormonal control. High concentrations of 2,4-D and low concentrations of kinetin in the induction medium promoted optimal shoot formation in tissue subsequently transferred to a regeneration medium. Conversely, low concentration of 2,4-D and high concentrations of kinetin promoted the subsequent formation of roots on a regeneration medium. It appears that a certain concentration of kinetin in the callus initiation medium was essential for induced callus to regenerate after transfer to a hormone-free regeneration medium (NAGARAJAN et al., 1986; KEYES & BINGHAM, 1979; BROWN & ATANASSOV, 1985).

Some studies showed that kinetin enhanced callus proliferation and regeneration by influencing mitosis, cytokinesis, total protein synthesis, lignin biosynthesis, vascular differentiation, the differentiation of mature chloroplasts from protoplastids, etc. (SZWEYKOWSKA, 1974).

Provious studies (PROFUMO, 1985, TANG et al., 1980) on the relationship between kinetin and regeneration suggested that calluses induced on kinetin-containing media had a characteristic morphology and histology which indicated their regenerability. The calluses induced on kinetin-containing medium were usually compact with small cells and nodular structures.

In this paper, we report the results of an investigation on the effects of kinetin on callus initiation, morphology, histology, and regenerability.

MATERIALS AND METHODS

We studied five F1 populations, two BC1 populations and two S1 populations (Table 1).

The plants were maintained in a greenhouse. Petioles of the second or third leaves from the stem apex were surface sterilized in 75% ethanol for 15 seconds, 5% commercial bleach for 5 minutes, and then washed three times in sterile distilled water. For callus initiation, explants of each plant were cut into pieces (about 0.5 cm long) and cultured on three initiation media: 1) 7951 (LIANG et al., 1982), 2) B2 (SAUNDERS & BINGHAM, 1975), and 3) B2-k which has the same components as B2 except kinetin was omitted. Media were maintained in Petri dishes. Cultures were placed in a growth chamber at 25 ± 1 C without light. Time required for callus initiation of each plant culture was recorded. After cultures were in the initiation media one month, callus size (width in mm), shape, and color were recorded. Calluses from three callus initiation media, chosen at random, were examined via a microscope.

In this paper, calluses obtained on the three media will be titled B2-k callus, B2 callus, and 7951 callus.

For experimental purposes, all calluses were transferred to the SHAP medium, which is a modified Schenk and Hildebrandt (1972) medium without hormone and with an addition of 50 um proline and 30 um alanine. Cultures were maintained in the growth chamber without light until they showed embryogenesis or bud initiation. Dishes with calluses showing regeneration were moved to a growth chamber with a 12-hour photoperiod at 25 ± 1 C.

Results were obtained from two replications. Each plant showing regeneration in either replication was recorded as a regenerable plant.

RESULTS AND DISCUSSIONS

Initiation and growth of callus. The three callus initiation media (7951, B2, and B2-k) had significantly different effects on callus initiation and growth. For each population tested, the shortest time required for callus initiation was on B2-k (Table 2). Calluses were initiated from explants after average of 5 to 7 days on B2-k, 8 to 10 days on 7951, and 10 to 12 days on B2 (Table 2). Kinetin (2 mg/l) apparently retarded callus initiation because B2 and B2-k are equal except for this component. 7951 contains 2 mg/l kinetin and also differs from B2 and B2-k quantitatively and qualitatively in inorganic and organic components. The retarding effect of kinetin on callus initiation may have been partially counteracted by other

components, because the time required for callus initiation on 7951 was shorter than that on B2 but longer than on B2-k.

Once initiated, calluses grew faster on 7951 and B2 than on B2-k. After 1 month on initiation media, calluses on B2 and 7951 were larger than those on B2-k (Table 3). Since calluses on B2-k were friable with loose structure, the small callus sizes were due only to slower divisions of callus cells. Similar results were obtained by Reynolds (1986) in callus cultures of Solanum carolinense and by Sun & Lu (1984) in rice anther culture. In Reynolds's study, kinetin was not necessary for callus initiation, but it enhanced proliferation. Sun and Lu (1984) found that callus initiation from rice anthers was retarded by the presence of kinetin. Kinetin apparently does not facilitate cell dedifferentiation and may block it so that callus initiation is retarded.

Morphology and histology of callus. Calluses induced on B2-k differed morphologically from those on 7951 or B2. The B2 and 7951 calluses were similar. Calluses induced on B2-k were usually small and friable with a smooth surface, while those on B2 and 7951 were larger and more compact with a rough surface and many densely packed structures resembling nodules.

Calluses induced on B2-k were separated easily into individual cells in stain solution (1% acetocarmine). The

cells were large, long, contained little cytoplasm, and stained faintly (Fig. 1). Calluses on B2 and 7951 were tough, cells were small, round, stained dark, and usually existed in aggregations(Fig. 2). Similar results were reported by Profumo (1985) for Cichorium intybus. Calluses induced on a kinetin-containing medium were hard with small cells, while those on 2,4-D medium lacking kinetin were highly friable with loose, large cells. Tang et al. (1980) found that calluses grown on a medium containing kinetin were tight and firm and eventually formed plantlets in tissue culture of Cucumis melo.

Callus type and callus regenerability were correlated. This corroborates the results obtained with other genera (Dale et al., 1981; Thomas et al., 1977; Vasil & Vasil, 1981). Their embryogenic calluses were firm, opaque, and had a nodular appearence.

Regenerability of callus. Calluses, induced on the three initiation media were transferred to a common regeneration medium, SHAP. Regenerability of calluses derived from the same plant were affected only by the initiation media. More plants were regenerable on SHAP from 7951 callus and B2 callus than from B2-k callus (Table 1 & Fig. 3). This was true for six (II, III, V, VI, VII and IX) of the nine populations. If the B2-k callus regenerated on SHAP, the B2 callus and 7951 callus, induced from the explants of the

same plant, also regenerated, but usually not vice versa. This indicated that: 1) callus induced on kinetin-free medium might lose its in vitro regenerability or 2) the presence of kinetin allowed the expression of the genetic potential on in vitro regeneration.

Generally, kinetin has enhanced cell differentiation (Szweykowska, 1974). Since kinetin was added in the callus initiation medium and not in the regeneration medium, it may have affected regeneration two ways. First, kinetin might have induced callus cell division to form growth centers and the growth centers progressively differentiated to form meristematic regions. The regeneration potential was established but these calluses expressed their potential only on regeneration medium or hormone free medium. Secondly, calluses, induced on kinetin-containing medium, might have higher concentration of kinetin (either absorbed from medium or induced by exogenous kinetin) or have a substance induced by kinetin. After these calluses were transferred to a hormone free medium, callus cells were induced to differentiate and regenerate plantlets through either embryogenesis or organogenesis. B2 callus and 7951 callus contained many cell aggregations similar to meristematic regions. Some vascular elements were observed in these regions (Fig. 4). Possibly the regeneration potential was established in the initiation medium

containing kinetin.

Embryoids developing to globular, heart, torpedo or cotyledon stage were observed on B2 callus and 7951 callus derived from some plants before these calluses were transfered to SHAP (Fig. 5). No plantlets were obtained from these embryoids, however, unless they were transfered to the hormone free medium, SHAP. This was due to: 1) recallusing of the embryoids (Fig. 6); 2) suppression by the proliferation of surrounding callus; 3) growth of embryoids stopped automatically. No embryoids were observed on B2-k callus before transfer to SHAP. This suggested that the regeneration potential of callus was established in the initiation medium and only in the presence of kinetin. Differentiated growth regions (embryoids) initiated growth, but failed to develop plantlets, because 2,4-D, inhibited regeneration processes (Stanis et al., 1983). In alfalfa callus culture, some researchers (Saunders & Bingham, 1975; Walker et al., 1978) also noted the formation of buds or shoots in callus tissue cultured on a callus initiation medium containing 2,4-D and kinetin, but plantlets failed to develop.

In Populations I, IV and VIII, the number of plants regenerated through B2-k was equal to the number regenerated through B2 or 7951. For these populations, kinetin didn't affect regeneration. Ladak-1 was a common parent of these populations, In our previous study (Wan et

al., in press), Ladak-1 was the only parental plant that regenerated through B2-k. The performance of the F1 and S1 plants was significantly affected by this parent. By comparing Population IV and V, we noted a reciprocal effect. When Ladak-1 was the male parent, fewer F1 plants regenerated through B2-k than when it was the female parent. This suggests a cytoplasm effect. Since alfalfa has self incompatability genes, a reciprocal effect could result from them.

The plants which regenerated when their calluses were induced on a kinetin-containing medium or kinetin-free medium were cytokinin (kinetin) autotrophic. In callus culture of tobacco cytokinin autotrophic callus tissue developed from calluses previously cultured on media with auxin and cytokinin and from tissues explanted directly onto media devoid of exogenous cytokinins (Kerbauy et al., 1986). Our data suggested that cytokinin (kinetin) autotrophism was sexually transmissable.

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Table 1. Effect of callus initiation medium on regeneration of plants.

Population and parent(s)	No. plants tested	No. plants regenerable through B2-k	B2	7951
I Ladak-1xLadak-6	35	17	17	26
II Ladak-28xLadak-12	44	8	36	42
III Ladak-6xLahontan-17	9	0	3	3
IV Ladak-1xLadak-42	11	9	9	11
V Ladak-42xLadak-1	45	16	37	40
VI H-127xLadak-6	19	5	13	12
VII H-127xLadak-12	29	7	28	26
VIII S1 of Ladak-1	17	9	6	13
IX S1 of Ladak-12	7	1	7	7

Table 2. Effect of callus initiation media on callus initiation.

Population	Time (days) requied for callus initiation					
	B2-k		B2		7951	
	Mean	Range	Mean	Range	Mean	Range
I	6.5	5--7	11.2	9--15	8.8	7--12
II	5.1	4--6	9.9	6--14	7.8	6--10
III	6.1	5--7	11.5	9--16	9.6	7--12
IV	5.8	5--7	12.0	10--15	9.1	7--11
V	6.1	5--8	11.5	8--15	8.9	7--13
VI	6.9	6--10	11.1	7--15	9.0	8--11
VII	6.3	5--9	11.7	8--16	8.8	6--12
VIII	6.4	5--11	10.6	8--14	9.1	6--12
IX	6.5	6--7	10.7	9--11	9.0	7--11

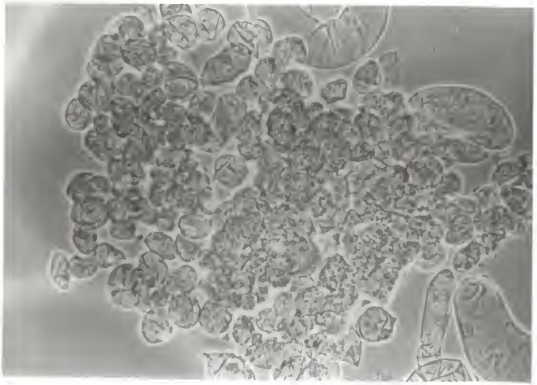
Table 3. Distribution of plants in callus size groups on three initiation media.

Population	No. plants tested	No. plants in callus groups* 1--2			2--3			3--4		
		B2-k	B2	7951	B2-k	B2	7951	B2-k	B2	7951
I	35	5	30	30	29	4	5	1	1	0
II	44	7	27	42	35	16	2	2	1	0
III	9	1	8	9	8	1	0	0	0	0
IV	11	5	8	9	5	3	2	1	0	0
V	45	34	34	38	11	11	7	0	0	0
VI	19	2	11	16	17	8	3	0	0	0
VII	29	5	11	25	23	17	4	1	1	0
VIII	17	7	16	13	9	1	4	1	0	0
IX	7	0	2	3	0	2	3	7	3	1

* 1 > 5mm, 2 = 4 to 5mm, 3 = 3 to 4mm, 4 < 3mm.
Plants were classified on the basis of two replications. Callus size differences between replications varied up to one class so each group includes two classes.

Fig. 1. Easily separated cells in B2-k callus.

Fig. 2. Cells and cell aggregation in B2 and 7951 calluses.



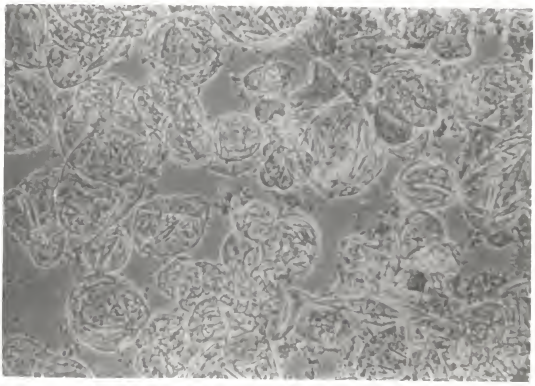


Fig. 3. Difference of callus regenerability among B2-k callus, B2 callus and 7951 callus.

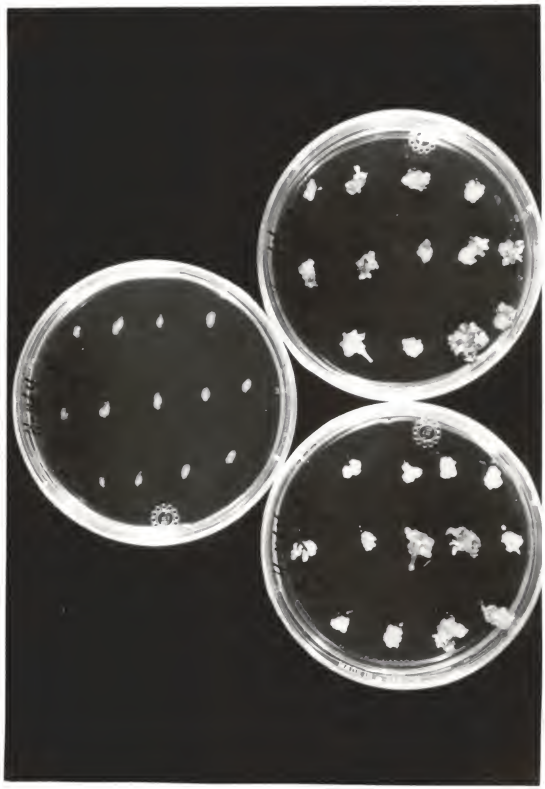


Fig. 4. Structure of 7951 callus developing vascular elements.

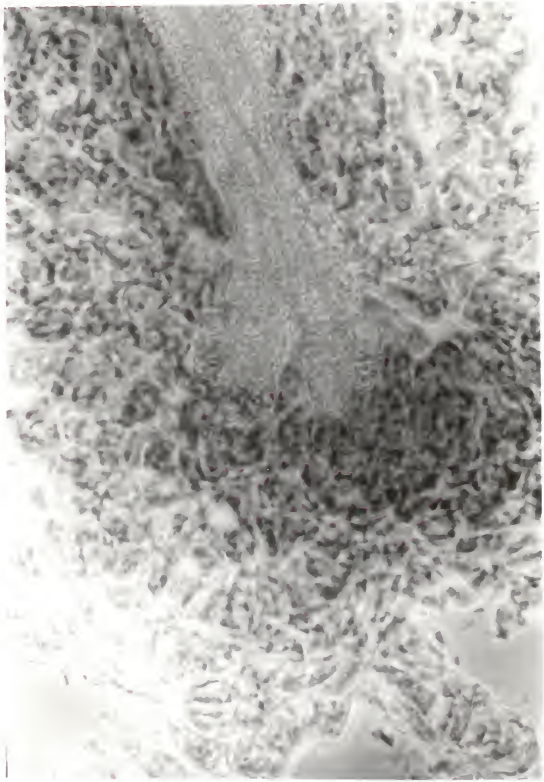


Fig. 5. Embryoids developed on B2 callus before transfer to regeneration medium.

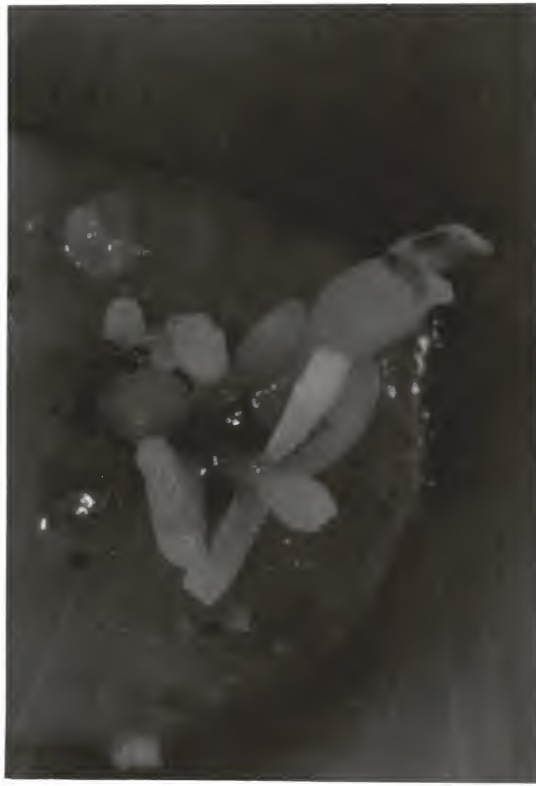
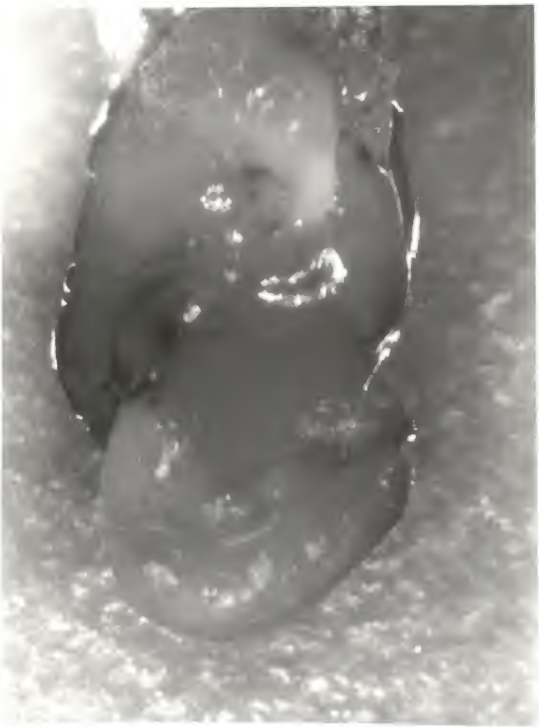


Fig. 6. Recallusing of a embryoid developed on 7951 callus before transfer to regeneration medium.



GENETIC CONTROL OF *IN VITRO* PLANT REGENERATION AND EFFECTS OF KINETIN ON CALLUS CHARACTERS IN ALFALFA (*MEDICAGO SATIVA* L.)

by

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ABSTRACT

The genetic control of plant regeneration from callus culture was studied in tetraploid alfalfa (Medicago sativa L.). Seven cultivars (total 72 plants) were screened for regenerability. Ladak had the best regeneration response, in which 42% of the plants regenerated. Four regenerable plants and three nonregenerable plants were used to form 10 F1 hybrids and three S1 populations. Segregation ratios in the populations suggested that regenerability of alfalfa via petiole culture was under the control of two complementary genes, Rn1 and Rn2. The presence of both dominant genes was necessary for a plant to regenerate in a two-step culture system. The data also indicated that gene dosage influenced regeneration efficiency. Significant reciprocal effects demonstrated that the interaction between callus induction medium and callus regenerability was affected by cytoplasmic factor(s).

Three callus induction media, B2-k, B2, and 7951, were used to study the effects of kinetin on callus initiation, morphology, histology, and regenerability. The presence of kinetin in callus induction media retarded callus initiation, but enhanced division and differentiation of callus cells. Calluses induced on kinetin-containing media (B2 and 7951) had many compact cell aggregations, which were considered meristematic regions that might

differentiate to plantlets on a regeneration medium. Visually, these calluses were compact and had many nodular structures. In contrast, most calluses induced on a kinetin-free medium were composed of larger, individual cells and had friable structures without nodules. After transfer to a hormone-free medium, calluses induced on kinetin-containing media regenerated more frequently than those induced on a kinetin-free medium, but cytokinin (kinetin) autotropism also occurred. Autotropism was transmitted sexually and especially affected by the female parent.