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# The effects of kinetin on callus characters in alfalfa (*Medicago sativa* L.)

Y. Wan, E.L. Sorensen and G.H. Liang
*USDA-ARS, Dept. of Agronomy, Throckmorton Hall, Kansas State University, Manhattan, KS 66506, U.S.A.*

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**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 in alfalfa (*Medicago sativa* L.). 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, has 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 and retarded callus formation and growth *in vitro,* but 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 kinetin, either alone or with 2,4-D or TIBA (Zheng et al., 1983).

In alfalfa *Medicago sativa* L. 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 it 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 concentra-

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tions of kinetin in the induction medium promoted optimal shoot formation in tissue subsequently transferred to a regeneration medium. Conversely, low concentrations of 2,4-D and high concentrations of kinetin promoted the subsequent formation of roots on a regeneration medium. It appeared that a certain concentration of kinetin in the callus initiation medium was essential for callus to regenerate plants 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).

Previous studies (Profumo et al., 1985, Tang et al., 1980) on the relationship between kinetin and regeneration suggested that calluses induced in 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 in nine alfalfa populations.

## Materials and methods

We studied five $F_1$ populations, from crosses Ladak-1 × Ladak-6, Ladak-28 × Ladak-12, Ladak-6 × Lahontan-17, Ladak-1 × Ladak-42, Ladak-42 × Ladak-1, two $BC_1$ populations from H-127 × Ladak-6 and H-127 × Ladak-12 and two $S_1$ populations from selfing of Ladak-1 and Ladak-12, respectively, in alfalfa (*Medicago sativa* L.).

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 with kinetin (Liang et al., 1982), 2) B2 with kinetin (Saunders & Bingham, 1975), and 3) B2-k with the same components as B2, but no kinetin. Media were maintained in Petri dishes (100 × 15 mm). 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 had been in the initiation media for one month, callus size (width in mm), shape, and color were recorded. Calluses from three callus initiation media, chosen at random, were examined microscopically.

Calluses obtained on the three media were designated 7951 callus, B2 callus, and B2-k callus.

For experimental purposes, all calluses were transferred to the SHAP medium which is a modified Schenk & Hildebrandt (1972) medium without hormone and with an addition of 50 $\mu$m proline and 30 $\mu$m 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.

Regenerability of each plant was tested two times. They were considered as two replications. Each plant showing regeneration in either replication was recorded as a regenerable plant.

## Results and discussion

*Initiation and growth of callus.* For each population tested, the shortest time required for callus initiation was on B2-k (Table 1). Calluses were initiated from explants after an average of 5 to 7 days on B2-k, 8 to 10 days on 7951, and 10 to 12 days on B2 (Table 1). Chi-square test showed that the three callus initiation media (7951, B2, and B2-k) had significantly different effects on callus initiation and growth. Kinetin apparently retarded callus initiation because B2 and B2-k are equal except for this component. Medium 7951 contains 0.5 mg/l kinetin and also differs from B2 and B2-k quantitatively and qualitatively in inorganic and organic

components. Since the significant difference in time required for callus initiation on B2-k and B2 was caused by kinetin, and the time required for callus initiation on 7951 was shorter than that on B2, but longer than on B2-k, it seems that 0.5 mg/l kinetin retarded callus initiation less than 2 mg/l, i.e., the retarding effect of kinetin on callus initiation was also influenced by the quantity of kinetin.

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 2). 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' study, kinetin was not necessary for callus initiation, but it enhanced proliferation. Sun & 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.

*Table 1.* Effect of callus initiation media on callus initiation.

| Population | Time (days) required for callus initiation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | B2-k | | B2 | | 7951 | | Chi-square | | |
| | Mean | Range | Mean | Range | Mean | Range | DF | Value | P |
| I | 6.5 | 5–7 | 11.2 | 9–15 | 8.8 | 7–12 | 20 | 243.1 | 0.000 |
| II | 5.1 | 4–6 | 9.9 | 6–14 | 7.8 | 6–10 | 20 | 361.9 | 0.000 |
| III | 6.1 | 5–7 | 11.5 | 9–16 | 9.6 | 7–12 | 18 | 69.9 | 0.000 |
| IV | 5.8 | 5–7 | 12.0 | 10–15 | 9.1 | 7–11 | 20 | 95.5 | 0.000 |
| V | 6.1 | 5–8 | 11.5 | 8–15 | 8.9 | 7–13 | 20 | 329.6 | 0.000 |
| VI | 6.9 | 6–10 | 11.1 | 7–15 | 9.0 | 8–11 | 18 | 117.2 | 0.000 |
| VII | 6.3 | 5–9 | 11.7 | 8–16 | 8.8 | 6–12 | 22 | 195.8 | 0.000 |
| VIII | 6.4 | 5–11 | 10.6 | 8–14 | 9.1 | 6–12 | 18 | 89.6 | 0.000 |
| IX | 6.5 | 6–7 | 10.7 | 9–11 | 9.0 | 7–11 | 14 | 53.2 | 0.000 |

*Table 2.* 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 = >5.0 mm, 2 = 4.0 to 4.9 mm, 3 = 3.0 to 3.9 mm, 4 = <3.0 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.

*Morphology and histology of callus.* Calluses induced on B2-k differed morphologically from those on 7951 or B2, which were similar. Calluses induced on B2-k were usually small and friable with a smooth surface, whereas 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, and cells were small, round, stained darkly, and usually existed in aggregations (Fig. 2). Similar results were reported by Profumo et al. (1985) for *Cichorium intybus.* Calluses induced on a kinetin-containing medium were hard with small cells, whereas those on 2,4-D medium lacking kinetin were highly friable with loose, large cells. In tissue culture of *Cucumis melo,* Tang et al. (1980) found that calluses grown on a medium containing kinetin were tight and firm and eventually formed plantlets.

Callus type and callus regenerability were related. 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 appearance.

*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 was affected only by the initiation media. More plants were regenerable on SHAP from 7951 callus and B2 callus than from B2-k callus (Table 3 & 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 in 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 to the callus initiation medium and not in the regeneration medium, it might have affected regeneration two ways. First, kinetin might have induced callus cell division to form growth centers, which progressively differentiated to form meristematic regions. The regeneration potential was established but these calluses expressed their potential only on regeneration (hormone-free) medium. Secondly, calluses, induced in the kinetin-containing medium might have had higher concentrations of kinetin (either absorbed from the medium or induced by exogenous kinetin) or of 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

*Table 3.* 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-1 × Ladak-6 | 35 | 17 | 17 | 26 |
| II | Ladak-28 × Ladak-12 | 44 | 8 | 36 | 42 |
| III | Ladak-6 × Lahontan-17 | 9 | 0 | 3 | 3 |
| IV | Ladak-1 × Ladak-42 | 11 | 9 | 9 | 11 |
| V | Ladak-42 × Ladak-1 | 45 | 16 | 37 | 40 |
| VI | H-127 × Ladak-6 | 19 | 5 | 13 | 12 |
| VII | H-127 × Ladak-12 | 29 | 7 | 28 | 26 |
| VIII | $S_1$ of Ladak-1 | 17 | 9 | 6 | 13 |
| IX | $S_1$ of Ladak-12 | 7 | 1 | 7 | 7 |

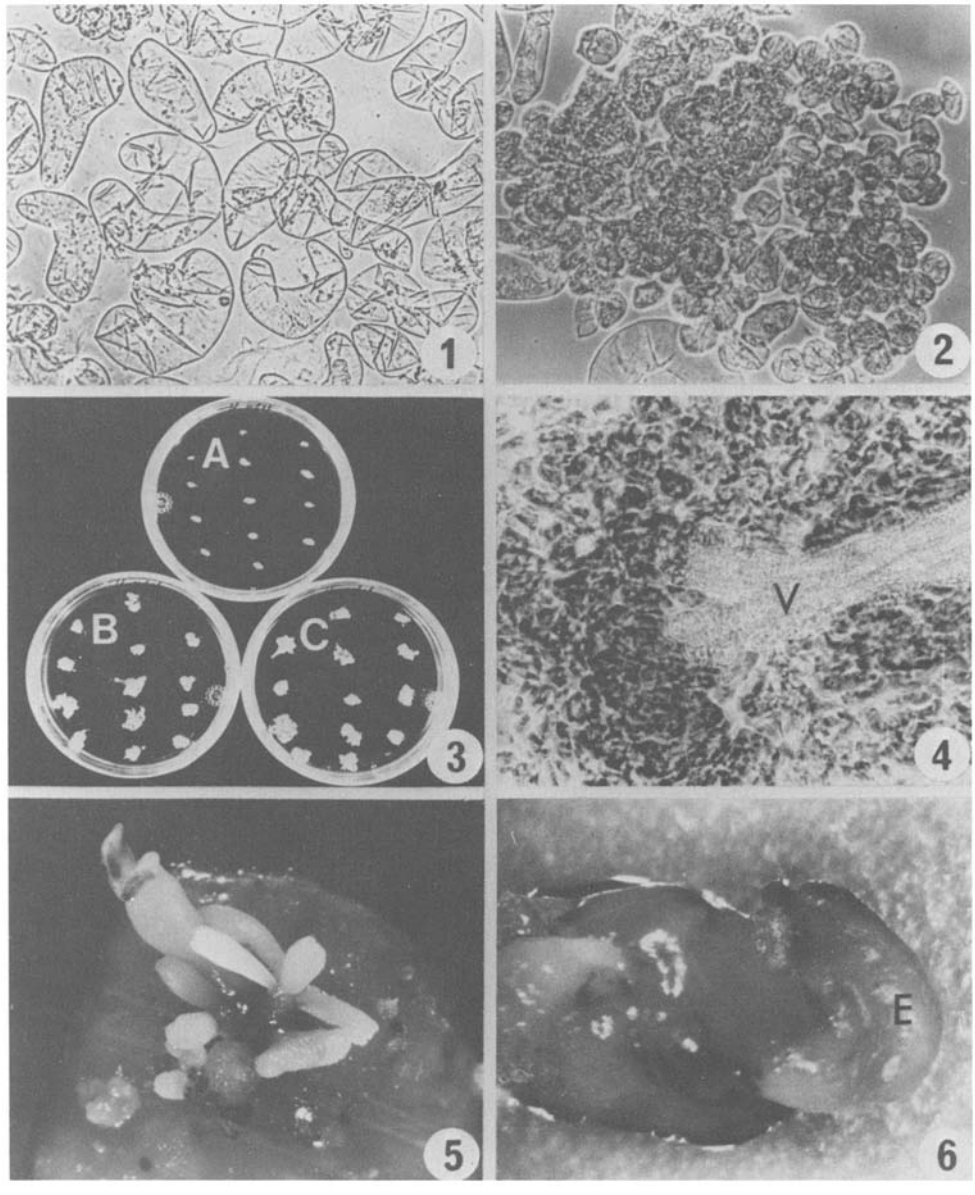


*Fig. 1.* Easily separated cells in B2-k callus of alfalfa.
*Fig. 2.* Cells and cell aggregation in B2 and 7951 calluses of alfalfa.
*Fig. 3.* Difference of callus regenerability among B2-k callus (A), B2 callus (B), and 7951 callus (C) of alfalfa.
*Fig. 4.* Structure of 7951 callus of alfalfa developing vascular (V) elements.
*Fig. 5.* Embryoids developed on B2 callus of alfalfa before transfer to regeneration medium.
*Fig. 6.* Recallusing (E) of a embryoid developed on 7951 callus of alfalfa before transfer to regeneration medium.

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 transferred to SHAP (Fig. 5). No plantlets were obtained from these embryoids, however, unless they were transferred to the hormone-free medium, SHAP. This was due to: 1) recallusing of the embryoids (Fig. 6); 2) suppres-

sion by the proliferation of surrounding callus; 3) immediate cessation of embryoid growth. 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 the presence of kinetin was necessary. 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, other researchers (Saunders & Bingham, 1975; Walker et al., 1978) also noted the formation of buds or shoots in callus tissue cultured on an 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 or larger than the number regenerated through B2 or 7951. For these populations, kinetin did not affect regeneration. Ladak-1 was a common parent of these populations. In our previous study, Ladak-1 was the only parental plant that regenerated through B2-k. The performance of the $F_1$ and $S_1$ 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 $F_1$ plants regenerated through B2-k than when it was the female parent. This suggests a cytoplasm effect. Since alfalfa has self-incompatibility genes, they could cause a reciprocal effect.

The plants that 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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