Effect of cytokinins on morphological, physiological and biochemical characteristics of shoots of citrus *in vitro*

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Effect of cytokinins on morphological, physiological and biochemical characteristics of shoots of citrus *in vitro*.

Abstract — **Introduction**. Cytokinins enhance axillary shoots, which helps prolific shoot multiplication *in vitro*. Very few reports are available on biochemical changes associated with this *in vitro* proliferation of *Citrus*. The present study was undertaken to find out the influence of two widely used cytokinins on various morphological and biochemical constituents of *in vitro* proliferated shoots of *Citrus* species. **Materials and methods**. Shoots of (0.5 to 1.0) cm long from *in vitro* proliferated shoots of five *Citrus* species were cultured on MS medium supplemented with eight increasing levels of benzylaminopurine (BAP) or kinetin spread from (0.25 to 2) mg·L^{-1}. After 6 weeks of culture, morphogenetic characters were observed, and shoot samples were crushed with 5 mL of hot ethanol; the extract collected was used for the estimation of reducing sugars, total phenols, ortho dihydric (OD) phenol, amino nitrogen and protein percentages. **Results and discussion**. For all the morphogenetic characters studied, except the shoot number, there were significant interactions between the *Citrus* species and the different concentrations of BAP and kinetine. Increased concentration of cytokinins reduced the amount of total phenol, OD phenol and total protein. Calamondin recorded the lowest percent of reducing sugar and protein. The maximum amount of reducing sugars was recorded for *C. volkameriana*. Supplementation of BAP resulted in the highest amount of total and OD phenols, whereas kinetin improved the reducing sugar and amino nitrogen.

India / *Citrus* / *in vitro* culture / plant growth substances / cytokinins / kinetin / 6-benzyladenine / biological development / chemical composition

Effet de cytokinines sur les caractéristiques morphologiques, physiologiques et biochimiques de vitroplants d'agrumes.

Résumé — **Introduction**. Les cytokinines augmentent la production de tiges axillaires, ce qui favorise la multiplication accélérée *in vitro* d'agrumes. Très peu de rapports existent sur les changements biochimiques associés à cette prolifération *in vitro* chez les *Citrus*. L'étude présentée a été entreprise pour mettre en évidence l'influence de deux cytokinines fréquemment utilisées, sur diverses caractéristiques morphologiques et biochimiques de vitroplants d'agrumes issus de prolifération. **Matériel et méthodes**. Des fragments de tige de (0,5 à 1,0) cm de long issus de vitroplants de cinq espèces de *Citrus* ont été cultivés sur milieux MS additionnés de benzylaminopurine (BAP) ou de kinétine utilisées à huit concentrations croissantes échelonnées de (0,25 à 2) mg·L^{-1}. Après 6 semaines de culture, certains caractères morphogénétiques ont été observés et des échantillons de tige ont été écrasés avec 5 mL d'éthanol chaud ; l'extrait obtenu a été utilisé pour évaluer les teneurs en sucres réducteurs, phénols totaux, phénol ortho-dihydrique (OD), azote aminé et protéine. **Résultats et discussion**. Pour tous les caractères morphogénétiques étudiés, sauf pour le nombre de tiges, il y a eu des interactions significatives entre espèces de *Citrus* étudiées et concentrations de BAP et kinétine utilisées. La plus forte concentration en cytokinines a réduit la quantité de phénol total, de phénol OD et de protéine totale. Le calamondin a enregistré les plus bas pourcentages de sucres réducteurs et de protéine. La quantité maximale de sucres réducteurs a été enregistrée pour *C. volkameriana*. L'addition de BAP a provoqué le plus fort taux de phénols OD et totaux, tandis que l'ajout de kinétine a amélioré le taux en sucres réducteurs et en azote aminé.

Inde / *Citrus* / culture *in vitro* / substance de croissance végétale / cytokinine / kinétine / 6-benzyladénine / développement biologique / composition chimique

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1. Introduction

Cytokinins enhance axillary shoots, which helps prolific shoot multiplication *in vitro*. Many workers have reported the successful use of cytokinins in *Citrus* micropropagation [1–4], but none of the reports contain any information on the biochemical and physiological changes caused by cytokinins. During the process, various biochemical changes in the shoot cultures result in reduced sugars and amino nitrogen concentration in proliferated cultures, besides physiological and morphological variations. Very few reports are available on biochemical changes associated with *in vitro* proliferation of *Citrus*. Hence, the present study was undertaken to find out the influence of two widely used cytokinins on various biochemical constituents of *in vitro* proliferated shoots of *Citrus* species.

2. Materials and methods

Shoots of (0.5 to 1.0) cm long from *in vitro* shoots of calamondin (*Citrus madurensis* Lour.), *C. volkameriana*, Malta orange (*C. sinensis* Osbeck.), Assam lemon (*C. limon* Burm.) and Troyer citrange (*Poncirus trifoliata* × *C. sinensis*) were cultured in Murashige and Skoog [5] medium (MS) supplemented with increasing levels of benzylaminopurine and kinetin, each at an increment of 0.25 mg·L^{-1} from (0.25 to 2) mg·L^{-1}. The control constituted MS medium without cytokinin. The details of culture conditions have already been reported [3, 6]. One shoot was cultured in each tube. Ten culture tubes formed one replication and the treatments were replicated twice.

After 6 weeks of culture, the morphogenetic characters – plant and leaf weight, number of shoots, leaves and nodes, and shoot length – were observed. Then, 1 g of sample was blended with 5 mL of hot ethanol; the extract and residue were used for estimation of biochemical constituents: reducing sugars [7], total phenols [8], ortho dihydric phenol, amino nitrogen [9] and protein [10].

The data were submitted to an ANOVA. Means were compared using the critical difference at $p = 0.05$.

3. Results and discussion

3.1. Effect of BAP and kinetin on *Citrus* morphogenetic characters

3.1.1. Effect of each cytokinin on each *Citrus* species

For all the morphogenetic characters studied except the shoot number, there were significant interactions between the *Citrus* species and the different concentrations of BAP and kinetine (*table I*).

In the case of calamondin, kinetin at 1.25 mg·L^{-1} resulted in the highest plant weight (0.3336 g). The number of shoots was found to be maximum in 0.50 mg·L^{-1} BAP, but shoots were significantly shorter compared to kinetin treatment. At a low concentration of BAP (0.25 mg·L^{-1}), more leaves were recorded, while the leaf weight was maximum (0.1020 g) at the highest level of kinetin (2.0 mg·L^{-1}). In *C. volkameriana* also, kinetin produced shoots with a higher plant weight (0.4282 g in 0.75 mg kinetin·L^{-1}). But, at the same concentration, BAP produced more shoots while kinetin produced longer shoots. The number of leaves was significantly higher at 0.75 mg BAP·L^{-1}, but the leaf weight was maximum in 1.0 mg kinetin·L^{-1}. For the Malta orange, positive responses of BAP were noticed for the plant weight, shoot number, leaf number, node number and leaf weight. A low concentration of BAP produced multiple shoots and their length was on a par with other kinetin levels. In Assam lemon, BAP was found to be the best for improving all attributes studied. Though 0.75 BAP mg·L^{-1} produced a maximum number of shoots, they were shorter than shoots produced by lower concentrations of BAP. For Troyer citrange, BAP was superior to kinetin for the morphogenetic characters studied and it was observed that 0.75 mg BAP·L^{-1} was the optimum.

Malta orange, Assam lemon and Troyer citrange recorded higher plant weight in

Table I.
Interaction between five *Citrus* species and two cytokinins added to a MS medium towards certain morphogenetic characteristics of shoots observed after 6 weeks of *in vitro* culture.

Citrus	Cytokinin	Plant weight (mg)	Number of shoots	Shoot length (cm)	Number of leaves	Number of nodes	Leaf weight (mg)
Calamondin	BAP	202.6	3.9	1.005	4.9	3.2	27.3
	Kinetin	217.0	1.3	2.077	3.9	3.2	61.6
Citrus volkameriana	BAP	205.0	3.3	1.256	5.0	4.6	46.7
	Kinetin	294.5	1.1	2.017	3.4	3.2	57.6
Malta orange	BAP	319.4	4.0	1.547	7.1	6.5	90.5
	Kinetin	108.0	1.3	1.081	3.9	3.4	25.9
Assam lemon	BAP	183.2	2.7	2.633	4.6	4.9	55.7
	Kinetin	119.7	1.1	2.411	3.5	2.8	43.9
Troyer citrange	BAP	298.8	9.8	1.469	7.1	7.1	78.5
	Kinetin	104.8	1.2	0.947	4.1	3.9	33.4
Standard error of the means	–	0.014	1.215	0.057	0.219	0.158	0.0046
Critical difference (p = 0.05)	–	0.039	3.368	0.158	0.607	0.438	0.0127

BAP, whereas kinetin produced shoots of maximum weight in calamondin and *C. volkameriana* (*table I*). In all the species, a maximum number of shoots was induced by BAP. In calamondin and *C. volkameriana*, longer shoots were produced by kinetin and, in other *Citrus*, BAP had a significant influence on shoot length. A wide variation was recorded for the number of leaves between BAP and kinetin. BAP produced more leaves and induced the maximum number of nodes in all species, except calamondin. A significantly higher node number (7.1) was obtained for Troyer citrange. Among the species, calamondin and *C. volkameriana* showed higher plant and leaf weights in kinetin, and the other *Citrus* in BAP. There was wide variation for leaf weight due to the effect of BAP and kinetin. Parthasarathy and Nagaraju [3] reported significant interaction between BAP × *Citrus* species, indicating the response of cytokinin varies between species.

The results on morphogenetic response of different *Citrus* species to varying concentrations of cytokinins (data not shown) indicated that the highest plant weight was recorded for calamondin at 0.50 mg·L^{-1}, whereas, for other species, 0.75 mg·L^{-1} was better. Similar results were obtained for the number of shoots but it was statistically non-significant. Cytokinin at all the levels produced shorter shoots than control. Cytokinin at 1.25 mg·L^{-1} recorded the highest shoot length in Malta orange and Troyer citrange. The number of leaves was superior in 0.75 mg cytokinin·L^{-1} for all the species, except calamondin, and it significantly differed. In calamondin, the maximum number of leaves was at 0.25 mg cytokinin·L^{-1}. The node number significantly differed between the species and between concentrations. At 1.50 mg cytokinin·L^{-1}, calamondin produced the most nodes, whereas, for *C. volkameriana* and Assam lemon, the optimum concentration was 0.50 mg cytokinin·L^{-1}. Malta orange and Troyer citrange recorded the maximum number of nodes in 0.75 mg cytokinin·L^{-1}. A lower concentration of BAP was found to be better for all variables of the shoot proliferation than higher concentrations of BAP and kinetin, irrespective of their concentrations. The highest value in plant weight was

recorded in 0.75 mg BAP·L^{-1}, followed by 0.50 mg BAP·L^{-1}. The lowest value was recorded in control media. Among BAP levels, 0.75 mg·L^{-1} was found to be the best for obtaining a good shoot number. There was significant difference for the shoot length and it was higher in control than in any other concentrations of BAP and kinetin. BAP produced more leaves than kinetin and 0.75 mg BAP·L^{-1} recorded the highest value. The node number and leaf weight were found to be maximum for the same concentration of BAP. Barua *et al.* [4] also observed that BAP was better than kinetin for pummelo (*C. grandis*).

3.1.2. Effect of cytokinins grouped on each *Citrus* species

When the mean response to BAP and kinetin was considered, there was significant difference between species for all the characters studied (*table II*). The highest value for plant weight was recorded for *C. volkameriana* (249.7 mg), and the lowest for Assam lemon (151.5 mg). The maximum number of shoots (5.5) and nodes (5.5) was produced by Troyer citrange, but, for this species, shoots were the shortest. The statistically longest shoots were recorded for Assam Lemon (2.5 cm). There were significant differences for the number of leaves and the leaf weight among the species. Troyer citrange produced the most leaves (5.6), but the leaf weight was the highest in Malta and the lowest in calamondin.

3.1.3. Effect of each cytokinin on all the *Citrus* species grouped

By considering the total response of the whole of the *Citrus* species to each of the cytokinins used, there were significant differences according to the addition of BAP or kinetin to the MS medium (*table III*); BAP showed a positive influence on all the characters except the shoot length, which was influenced by kinetin.

3.1.4. Effect of each concentration of cytokinin on all the *Citrus* species grouped

The different concentrations of cytokinin had a significant influence on all variables (*table IV*). A concentration at 0.75 mg cytokinin·L^{-1} resulted in the heaviest shoots (321.5 mg) and leaves (73 g), and the maximum number of shoots (7.9), leaves (6.6) and nodes (5.4). It was observed that cytokinin supplementation had no effect on the shoot length.

3.2. Effect of BAP and kinetin on *Citrus* biochemical constituents

3.2.1. Effect of each concentration of cytokinin on all the *Citrus* species grouped

When a cytokinin was added to the medium, the total phenol, ortho dihydric phenol and protein content were reduced (*table V*).

Table II.
Mean response of five *Citrus* species to BAP and kinetin added to a MS medium towards certain morphogenetic characteristics of shoots observed after 6 weeks of *in vitro* culture. All the results obtained with the different concentrations of the cytokinins (BAP + kinetin) are grouped.

Citrus	Plant weight (mg)	Number of shoots	Shoot length (cm)	Number of leaves	Number of nodes	Leaf weight (mg)
Calamondin	209.8	2.6	1.541	4.4	3.2	44.50
Citrus volkameriana	249.7	2.2	1.637	4.2	3.9	52.10
Malta orange	213.7	2.6	1.314	5.5	4.9	58.20
Assam lemon	151.5	1.9	2.522	4.1	3.8	49.80
Troyer citrange	201.8	5.5	1.208	5.6	5.5	55.90
Standard error of the means	0.0099	0.859	0.041	0.155	0.114	0.0033
Critical difference (p = 0.05)	0.0274	2.381	0.114	0.429	0.316	0.0092

Table III.
Morphogenetic response of shoots of *Citrus* observed after 6 weeks of *in vitro* culture, to the addition of BAP or kinetin to a MS medium. Results obtained with all the concentrations of each of the cytokinins are grouped. All the data obtained with the five *Citrus* species are grouped.

Cytokinin	Plant weight (mg)	Number of shoots	Shoot length (cm)	Number of leaves	Number of nodes	Leaf weight (mg)
BAP	241.8	4.8	1.582	5.7	5.3	59.7
Kinetin	168.8	1.2	1.707	3.8	3.3	44.5
Standard error of the means	0.0062	0.543	0.026	0.098	0.070	0.0021
Critical difference ($p = 0.05$)	0.0172	1.5051	0.072	0.272	0.194	0.0058

Table IV.
Effect of different growth concentrations of cytokinins on certain *Citrus* morphogenetic shoot characteristics, after 6 weeks of *in vitro* culture. Means of results obtained for each cytokinin (BAP + kinetin) concentration.

Cytokinin concentration ($mg \cdot L^{-1}$)	Plant weight (mg)	Number of shoots	Shoot length (cm)	Number of leaves	Number of nodes	Leaf weight (mg)
0.00	146.0	1.0	1.952	4.3	4.0	38.3
0.25	218.3	2.4	1.805	5.4	3.7	53.4
0.5	221.4	3.5	1.830	5.5	5.1	59.2
0.75	321.5	7.9	1.733	6.6	5.4	73.0
1.0	191.1	2.7	1.414	4.8	4.5	43.6
1.25	198.7	2.3	1.695	4.7	4.1	48.3
1.5	174.3	2.1	1.512	3.9	4.0	47.7
1.75	180.4	2.4	1.449	3.8	3.9	46.8
2.0	196.0	2.5	1.408	3.6	3.8	58.6
Standard error of the means	0.013	1.152	0.055	0.207	0.149	0.004
Critical difference ($p = 0.05$)	0.036	3.193	0.152	0.574	0.413	0.012

Table V.
Effect of different growth concentrations of cytokinins on certain *Citrus* shoot biochemical constituents (expressed in %), after 6 weeks of *in vitro* culture. Means of results obtained for each cytokinin (BAP + kinetin) concentration.

Cytokinin concentration ($mg \cdot L^{-1}$)	Reducing sugar	Total phenol	Ortho dihydric phenol	Amino nitrogen	Protein
0.00	0.316	0.006	0.015	0.014	0.292
0.25	0.271	0.017	0.021	0.008	0.215
0.50	0.204	0.022	0.026	0.006	0.265
0.75	0.173	0.019	0.027	0.009	0.326
1.00	0.224	0.015	0.026	0.008	0.226
1.25	0.450	0.011	0.018	0.012	0.234
1.50	0.269	0.010	0.016	0.03	0.188
1.75	0.325	0.009	0.018	0.072	0.208
2.00	0.415	0.008	0.019	0.016	0.209
Standard error of the means	0.077	0.002	0.002	0.002	0.029
Critical difference ($p = 0.05$)	ns	0.0056	0.0056	0.0056	0.0815

ns: not significant.

Table VI.
Mean response of five *Citrus* species to BAP and kinetin added to a MS medium towards certain biochemical variations (expressed in %) of *in vitro* proliferated shoots after 6 weeks of *in vitro* culture. All the results obtained with the different concentrations of the cytokinins (BAP + kinetin) are grouped.

Citrus	Reducing sugar	Total phenol	Ortho dihydric phenol	Amino nitrogen	Protein
Calamondin	0.227	0.013	0.022	0.013	0.201
Citrus volkameriana	0.421	0.010	0.017	0.011	0.247
Malta orange	0.245	0.011	0.021	0.013	0.299
Assam lemon	0.309	0.014	0.021	0.010	0.236
Troyer citrange	0.268	0.017	0.022	0.008	0.241
Standard error of the means	0.057	0.001	0.002	0.001	0.022
Critical difference (p = 0.05)	ns	0.0027	ns	0.0028	0.0618

ns: not significant.

Higher concentrations of cytokinin improved the amino nitrogen concentration. Such a variation among the proliferating shoots at various levels of cytokinin could be due to the utilization of the constituents by proliferating cultures for proliferation and growth. Rodriguez and Fernandez [11] also reported similar findings in the case of walnut. They found that phenolic compounds in walnut seeds decreased with BA in the medium.

A higher amount of sugars was noticed with cytokinin treatment as reported earlier by Zerbe and Wild [12] in the case of *Sinapis alba*.

3.2.2. Effect of cytokinins grouped on each *Citrus* species

The maximum amount of reducing sugar was recorded in *C. volkameriana*, while the amount of total phenol was low (*table VI*). Calamondin recorded the lowest percent of reducing sugar and protein. Malta orange recorded a high amount of protein. Troyer citrange recorded the highest amount of total phenol and the lowest amount of amino nitrogen. According to Hazarika *et al.* [6], significant differences of biochemical constituents could exist among the *Citrus* species, except for starch.

Table VII.
Effect of cytokinins added to a MS medium on biochemical constituents (expressed in %) of *in vitro* proliferated shoots of citrus, observed after 6 weeks of *in vitro* culture. Results obtained with all the concentrations of each of the cytokinins are grouped. All the data obtained with the five *Citrus* species are grouped.

Cytokinin	Reducing sugar	Total phenol	Ortho dihydric phenol	Amino nitrogen	Protein
BAP	0.228	0.018	0.023	0.005	0.241
Kinetin	0.360	0.008	0.019	0.016	0.241
Standard error of the means	0.036	0.001	0.001	0.001	0.014
Critical difference (p = 0.05)	0.100	0.0028	0.0028	0.0028	ns

ns: not significant.

3.2.3. Effect of each cytokinin on all the *Citrus* species grouped

By considering the effect of each cytokinin on all the *Citrus* species grouped, the medium with BAP allowed the recording of the highest percent of total and ortho dihydric phenol, whereas the addition of kinetin produced shoots with the highest amount of reducing sugar and amino nitrogen (*table VII*). The low amounts of reducing sugar and amino nitrogen in the shoots cultured on BAP supplemented media might be due to the utilization of these constituents by the proliferating shoots. For increasing the proliferation rate, the use of BAP is superior to kinetin. Proliferating cultures mainly utilize sugars and amino nitrogen. Parallel studies using paclobutrazol indicated the usefulness of these biochemical parameters on *Citrus* tissue culture [6].

There was a linear increase of the biochemical constituents, *viz*., reducing sugars, starch and phenol, with the addition of paclobutrazol to the medium [6, 13], wherein paclobutrazol treatment resulted in a shift of the partitioning of assimilates from the leaves to the roots, with an increase in carbohydrates, chlorophyll, soluble proteins and mineral element concentration in the leaf tissue.

Total phenol was also significantly increased with an increased concentration of paclobutrazol in the growth medium. This might be due to the high content of quinic acid occurring in paclobutrazol-treated plants [13]. Starch content also increased with the progressive increase in paclobutrazol concentration in the media from (0.0015 to 0.0047)%. Steffens *et al*. [14], Wang *et al.* [15], and Nagaraju [16] also reported similar findings. The increased starch content may be due to decreased starch hydrolysis because of reduced amylase activity in paclobutrazol-treated tissues [14, 17].

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Efecto de las citoquininas en las características morfológicas, fisiológicas y bioquímicas de vitroplantas de cítricos.

Resumen — **Introducción**. Las citoquininas aumentan la producción de tallos axilares, lo cual favorece la multiplicación acelerada *in vitro* de cítricos. Existen muy pocos informes acerca de los cambios bioquímicos asociados a esta proliferación *in vitro* en *Citrus*. Este estudio se acometió con el propósito de evidenciar la influencia de dos citoquininas, frecuentemente utilizadas, en diferentes características morfológicas y bioquímicas de vitroplantas de cítricos procedentes de proliferación. **Material y métodos**. Se cultivaron fragmentos de tallo (de 0,5 a 1,0 cm de longitud) procedentes de vitroplantas de cinco especies de *Citrus* en medios MS adicionados con bencilaminopurina (BAP) o con kinetina utilizadas con ocho concentraciones crecientes escalonadas de (0,25 a 2) $mg \cdot L^{-1}$. Tras 6 semanas de cultivo, se observaron algunos caracteres morfogenéticos y se machacaron muestras de tallos con 5 mL de etanol caliente; el extracto obtenido se utilizó para evaluar los contenidos en azúcares reductores, fenoles totales, fenol ortodihídrico (OD), nitrógeno amínico y proteína. **Resultados y discusión**. En todos los caracteres morfogenéticos estudiados, excepto en cuanto al número de tallos, hubo interacciones significativas entre las especies de *Citrus* estudiadas y las concentraciones de BAP y kinetina utilizadas. La concentración más fuerte de citoquininas redujo la cantidad de fenol total, de fenol OD y de proteína total. El calamondín registró los porcentajes más bajos de azúcares reductores y de proteína. La cantidad máxima de azúcares reductores se registró en *C. volkameriana*. La adición de BAP provocó la tasa más alta de fenoles OD y totales, mientras que el añadido de kinetina mejoró la tasa de azúcares reductores y nitrógeno amínico.

India / *Citrus* / cultivo *in vitro* / sustancias de crecimiento vegetal / citoquininas / quinetina / 6-benziladenina / desarrollo biológico / composición química

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