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RESEARCH ARTICLE

# Evaluation the Effect of Growth Regulators and Explants Source on Micro Propagation of *Moringa Oleifera In Vitro*

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## Abstract

The present study describes a procedure for indirect regeneration of *Moringa oleifera* using leaves and stems as explants derived from *in vitro* germinated seedlings grown on Murashige and Skoog (MS) medium supplemented with different concentrations of auxin and cytokinins. The best callus induction was observed on MS medium containing 1.5 mg.L$^{-1}$Indole-3-butyric acid (IBA) with 1.5 mg.L$^{-1}$6-benzylaminopurin (BAP) on stem explants .However, when callus was transferred onto a maintenance medium supplemented with different concentrations of IBA (0.0, 1.0, 1.5, 2.0 or 2.5) mg.L$^{-1}$and BAP (0.0, 1.0, 1.5, 2.0 or 2.5) mg.L$^{-1}$, for increasing callus mass, the combination of 1.5 mg.L$^{-1}$ IBA with 1.0 mg.L$^{-1}$ BAP resulted in best response in increasing callus fresh weight. A high shoot proliferation percentage was achieved at the combination 1.0 mg.L$^{-1}$Kinetin (Kin) with 0.4 mg.L$^{-1}$Naphthalene acetic acid (NAA) recorded (90%).The rooting percentage was 100% for shoots regenerated from callus cultures when MS medium was supplemented with 2.0 mg.L$^{-1}$ IBA .After acclimatization, 80% of plants were able to survive.

**Keywords:** *Moringa oleifera,* Indirect organogenesis, Explants, *In vitro,* Callus.

## Introduction

*Moringa oleifera* L. belongs to Moringaceae family which includes 12 other species. It has been called the Miracle tree or tree of Life, in reference to its potential medicinal use. It is very nutritious and has a variety of potential uses [1]. The tree is highly nutritive, drought resistant, grows very fast. It also offers many benefits for the third world countries particularly India, Pakistan, Philippines, Hawaii and many parts of Africa. It has been utilized in cleaning water [2].

Reports indicated that the plant contains various amino acids, fatty acids, vitamins, nutrients and its parts have been used frequently as herbal medicine [3]. The different parts of *M. oleifera* such as leaves, flowers, fruits and seeds are also known to be good sources for phytochemicals. [3]. *M. oleifera* leaves contain more vitamin A than carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas. The protein quality of Moringa is better than that of milk and eggs. Clonal propagation refers to asexual reproduction after multiplication of genetically identical copies of individual plants [4].

Organogenesis is the morphogenesis process that involving the formation of plant organs like shoots, roots, buds, flowers from explants or cultured plant tissues. There are two types of organogenesis direct and indirect organogenesis [5]. When organogenesis occurs through callus or cell suspension culture, it is called indirect organogenesis. In indirect organogenesis, the cultures may be established in liquid or solid media.

The growth regulators supplemented into the medium have important critical role in organogenesis [5]. Thus, the aim to this study is to establish a plant tissue culture protocol for indirect organogenesis in *M. Oleifera* using leaves and stems as explants.

## Methods

### Plant Source and Callus Induction

Seeds of *Moringa oleifera* were purchased from Agricultural Seeds and Medicinal Plants Company Cairo, Egypt. Seeds were washed under running tap water for five minutes. Surface sterilization of seeds were carried out inside a laminar air flow cabin ate by immersion in 0.0, 1.0, 1.5 or 2.0% (v/v) sodium hypochlorite for 5, 10 or 15 min, followed by washing three times in sterile distilled water.

The sterilized seeds were transferred to MS medium [6] and incubated at 25 ± 2 ºC, 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. After two weeks of incubation, percentages of germinated seeds were recorded. The explants (leaves and stems) of *M. oleifera* 4 cm long were cut and placed on MS medium supplemented with different concentrations of IBA (0.0, 1.0, 1.5, 2.0 or 2.5 $mg.L^{-1}$) and BAP (0.0, 1.0, 1.5, 2.0 or 2.5 $mg.L^{-1}$).

All cultures were incubated at 25 ± 2 ºC, 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. After four weeks of incubation, percentages of explants produced callus were recorded. Ten replicates per treatment were used, each replicate contained 3 explants, and the percentage of response to callusing was calculated according to [7]. Percentage of explants initiated callus = (No. of explants produced callus/ Total no. of cultured explants) x 100.

### Maintenance of Callus Cultures

For maintenance of callus, the light- yellow healthy callus weighting 40 mg was sub cultured onto MS medium supplemented with IBA (0.0, 1.0, 1.5, 2.0 or 2.5 $mg.L^{-1}$) and BAP (0.0, 1.0, 1.5, 2.0 or 2.5 $mg.L^{-1}$). Callus tissues were incubated at 25 ± 2 ºC, 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. After four weeks of incubation, callus fresh weight was recorded.

### Shoot Proliferation Medium

Depending on the previous results, callus was initiated on 1.0 $m.L^{-1}$ BAP and1.5 $mg.L^{-1}$ IBA. Callus pieces (100 mg) were transferred into the regeneration medium under aseptic conditions .MS medium supplemented with Kin (1.0, 1.5 2.0 or 2.5 $mg.L^{-1}$) and NAA (0.2, 0.3 or 0.4 $mg.L^{-1}$). All cultures were maintained at 25 ± 2 ºC for 16/8hrs (light/dark) photoperiod at a light intensity of 1000 lux. Number of regenerate shoots was recorded after three months of transfer [8].

### *In Vitro* Rooting

For in vitro rooting, 3-4 cm height of well proliferated shoots was transferred onto MS medium supplemented with2 $mg.L^{-1}$ IBA. All cultures were maintained as indicated above. The percentage of rooted shoots was calculated after one month of culture [9].

### Acclimatization of Plantlets

Plantlets (3-4 cm height) having fully expanded leaves and well- developed roots were removed from culture flasks and thoroughly washed with sterile distilled water to remove agar, then they transferred to pots contained sterilized mixture of soil and sand (1:1 v/v) then covered with clear white plastic bags. The plants were kept in a growth chamber. After two weeks, the plants were placed in a greenhouse. The plastic covers were removed after two weeks thus plants were fully exposed to the normal growth conditions and the survival rate was recorded after a month.

### Experimental design and statistical analysis

The experiments were designed as a completely randomized design (CRD) with ten replicates per treatment. Analyses were done using the SPSS statistical program ver. 9 software. Differences between means were determined and least significant differences were compared at probability level of 5%.

## Results and Discussion

### Callus Induction

Table 1 shows that addition of BAP to the culture medium at the concentration 1.0 $mg.L^{-1}$ led to a significant increase in response percentage of leaf explants showing callus induction up to 51.32% as compared with the control treatment which recorded 29.99%. Increasing BAP concentration led to a decrease in response percentage of callus induction compared with 1.0 $mg.L^{-1}$ BAP, achieving 39.98, 28.66 and 37.99% at 1.5, 2.0 and 2.5 respectively. Addition of IBA at the concentration of 2.0$mg.L^{-1}$ caused a significant increase in the percentage of leaf explants showing callus induction recording 63.99%.

 

The interaction between BAP and IBA at the combination of2.0 mg.L$^{-1}$ IBA and 1.0 mg.L$^{-1}$ BAP led to a significant increase in response percentage for callus induction achieved 86.66% as compared with control treatment (0.0%).

**Table 1: Effect of IBA or BAP and their interaction on leaf explants response (%) for callus induction after inoculation on MS medium for four weeks, n=10 (each replicate contained 3 explants)**

| IBA (mg.L$^{-1}$) / BAP (mg.L$^{-1}$) | 0.0 | 1.0 | 1.5 | 2.0 | 2.5 | Mean (%) |
|---|---|---|---|---|---|---|
| 0.0 | 0.0 | 13.33 | 53.33 | 60 | 23.33 | 29.99 |
| 1.0 | 0.0 | 73.3 | 43.33 | 86.66 | 53.33 | 51.32 |
| 1.5 | 0.0 | 26.6 | 43.33 | 63.33 | 66.66 | 39.98 |
| 2.0 | 0.0 | 30 | 20 | 36.66 | 56.66 | 28.66 |
| 2.5 | 0.0 | 16.66 | 33.33 | 73.33 | 66.66 | 37.99 |
| Mean (%) | 0.0 | 31.98 | 38.66 | 63.99 | 53.33 | --- |
| LSD 0.05 | IBA= 7.39 * , BAP= 7.39 * , IBA x BAP = 13.52 *. | | | | | |

Table 2 shows that the addition of BAP at the concentration 1.0 mg.L$^{-1}$ led to a significant increase in response percentage of stem explants showing callus induction achieving 57.32% as compared with the control treatment (41.99%). Increasing BAP concentration led to decrease the response percentage for callus induction compared with 1.0 mg.L$^{-1}$ BAP, achieving 52.66, 51.99 and 42.66% at 1.5, 2.0 and 2.5 respectively. Addition of IBA at 2.5mg.L$^{-1}$ led to a significant increase in the percentage of stem explants showing callus induction recording 73.98%. The interaction between BAP and IBA in the combination of 1.5 mg.L$^{-1}$ IBA and 1.5 mg.L$^{-1}$ BAP led to a significant increase in response percentage for callus induction on stem explants achieved 93.3% as compared with the control treatment which exhibited no response.

**Table 2: Effect of IBA or BAP and their interaction on stem explants response (%) for callus induction after inoculation on MS medium for four weeks, n=10 (each replicate contains 3 explants)**

| IBA (mg.L$^{-1}$) / BAP (mg.L$^{-1}$) | 0.0 | 1.0 | 1.5 | 2.0 | 2.5 | Mean (%) |
|---|---|---|---|---|---|---|
| 0.0 | 0.0 | 46.66 | 26.66 | 80 | 56.66 | 41.99 |
| 1.0 | 0.0 | 53.33 | 83.33 | 73.33 | 76.6 | 57.32 |
| 1.5 | 0.0 | 23.33 | 93.3 | 76.66 | 70 | 52.66 |
| 2.0 | 0.0 | 56.66 | 56.66 | 70 | 76.66 | 51.99 |
| 2.5 | 0.0 | 10 | 56.66 | 56.66 | 90 | 42.66 |
| Mean (%) | 0.0 | 37.99 | 63.32 | 71.33 | 73.98 | --- |
| LSD 0.05 | IBA= 8.74 * , BAP= 8.74 * , IBA x BAP = 15.62 *. | | | | | |

Establishment of a callus from explants was illustrated by [10] who divided the process into three phases including induction, cell division and differentiation phase. The period of these phases depends on the physiological status of the explants cells and culture conditions such as appropriate combination of plant growth regulators. Variation in callus induction might be the results endogenous hormone presence in explants in addition to those supplemented to the medium. Calli varied not only in weights, but also in morphology such as color, they could be green, white or yellow sh.

These results are similar to those obtained by [11] and [12] who reported that a high callus induction % was achieved by using M. *oleifera* stems as explants cultured on MS medium supplemented with equal concentrations of 1.5 mg/L$^{-1}$ IBA and BAP. Results of the present study also showed that explants cultured on a medium containing IBA responded well for call using. Using BAP alone has not been promising in both leaf and stem explants. IBA induced cell division since it is among the widely used auxins for *in vitro* callus induction [13]. *M. oleifera* stem explants may contain certain levels of endogenous auxins that made a balanced ratio with the exogenous auxins and may be even cytokinins.

These results are similar to those obtained by [14] who reported that MS medium supplementation with IBA in combination with BAP gave the highest response percentage of callus induction on *M. oleifera* explants. It is concluded from the current study that the callus masses formed on stem explants cultured on MS medium supplemented with 1.5 mg.L$^{-1}$ IBA and BAP

 

are larger than those formed on the leaf explants. Callus masses initiated on stem explants were used for maintenance of callus cultures in this study.

## Effect of IBA and BAP on Mean Callus Fresh Weight

Table 3 revealed that the addition of IBA exhibited a positive effect on *M. oleifera* callus growth at the concentration of 1.5mg.L$^{-1}$ in combination with 1.0 mg.L$^{-1}$ BAP. Inclusion of IBA at the concentration of 1.5 mg.L$^{-1}$ gave significantly higher callus fresh weight (195.6) mg than other concentrations, while the lowest fresh weight (0.00) mg was obtained in IBA free medium. The highest callus fresh weight obtained from BAP treatment (233.8) mg occurred at the concentration 1.0 mg.L$^{-1.}$ The interaction between IBA and BAP at the addition 1.5 mg.L$^{-1}$ IBA and 1.0 mg.L$^{-1}$ BAP (Figure 1) resulted in maximum callus fresh weight (747.0) mg, this treatment was significantly higher than other combinations.

**Table 3: Callus fresh weight (mg) grown on MS medium supplemented With different combinations of IBA and BAP, after four weeks, the initial callus fresh weight is 40 mg, (n=10)**

| IBA mg.L$^{-1}$ / BAP mg.L$^{-1}$ | 0.0 | 1.0 | 1.5 | 2.0 | 2.5 | Mean |
|---|---|---|---|---|---|---|
| 0.0 | 0.0 | 44.0 | 51.0 | 113.0 | 43.0 | 50.2 |
| 1.0 | 0.0 | 195.0 | 747.0 | 87.0 | 140.0 | 233.8 |
| 1.5 | 0.0 | 80.0 | 76.0 | 53.0 | 88.0 | 59.4 |
| 2.0 | 0.0 | 54.0 | 68.0 | 188.0 | 53.0 | 72.6 |
| 2.5 | 0.0 | 10.0 | 36.0 | 50.0 | 95 | 38.2 |
| Mean | 0.0 | 76.6 | 195.6 | 98.2 | 83.8 | --- |
| LSD 0.05 | IBA= 9.026 * , BAP= 9.026 * , IBA x BAP = 17.371 * | | | | | |

Mean callus fresh weight decreased significantly with the increasing IBA concentration recording 98.2 and 83.8 mg at 2.0 and 2.5mg.L$^{-1}$ IBA respectively. These results are in agreement with those of [15]. Who reported that *M. oleifera* callus fresh weight decreased significantly with the increasing auxin concentration? Callus grown on MS medium containing 0.5 mg.L$^{-1}$ 2,4-D increased in weight and size. These results disagree with those obtained by [16] who reported that M. *oleifera* callus fresh weight increased with increasing auxin concentration. The increase in callus mass was important for production of secondary metabolites since they are proportionally related [17].

**Figure 1: *M. oleifera* callus maintained on MS medium supplemented with 1.5mg.L$^{-1}$ IBA and 1.0 mg.L$^{-1}$ BAP after four weeks**

## Shoot Proliferation from Callus Cultures

Results displayed in table 4 show that Kin increased the shoot proliferation percentage significantly at 1.5mg.L$^{-1}$. Inclusion of Kin at the above concentration gave higher shoot proliferation percentage than the others reached 76.67%, while the lowest shoot regeneration percentage was obtained at the concentration 2.0 mg.L$^{-1}$. The highest shoot proliferation percentage obtained from NAA treatment (82.5%) occurred at the concentration 0.4 mg.L$^{-1}$, it is gave higher shoot proliferation percentage than others, while the lowest was obtained at the concentration 0.3 mg.L$^{-1}$. Results of the

 

interaction between Kin and NAA indicated that the a high shoot proliferation percentage was achieved at the combination 1.0 mg.L$^{-1}$ Kin with 0.4 mg.L$^{-1}$ NAA recorded (90%) compared with other combinations, and gave the maximum shoot proliferation percentage as shown in Figure 2**.** No significant differences were recorded among the treatments 1.0, 1.5, 2.0 and 2.5 mg.L$^{-1}$ of Kin in the presence of NAA at 0.4 mg.L$^{-1}$.

**Table 4: Effect of NAA and Kin and their interaction on mean % of shoot proliferation, after inoculating callus pieces for three months on MS medium, n=10**

| Kin (mg.L$^{-1}$) / NAA (mg.L$^{-1}$) | 1.0 | 1.5 | 2.0 | 2.5 | Mean (%) |
|---|---|---|---|---|---|
| 0.2 | 50 | 80 | 70 | 70 | 67.5 |
| 0.3 | 80 | 70 | 50 | 60 | 65.0 |
| 0.4 | 90 | 80 | 80 | 80 | 82.5 |
| Mean (%) | 73.33 | 76.67 | 66.67 | 70.00 | --- |
| LSD 0.05 | NAA = 5.92 * , Kin = 6.37 * , NAA x Kin = 11.07 * | | | | |

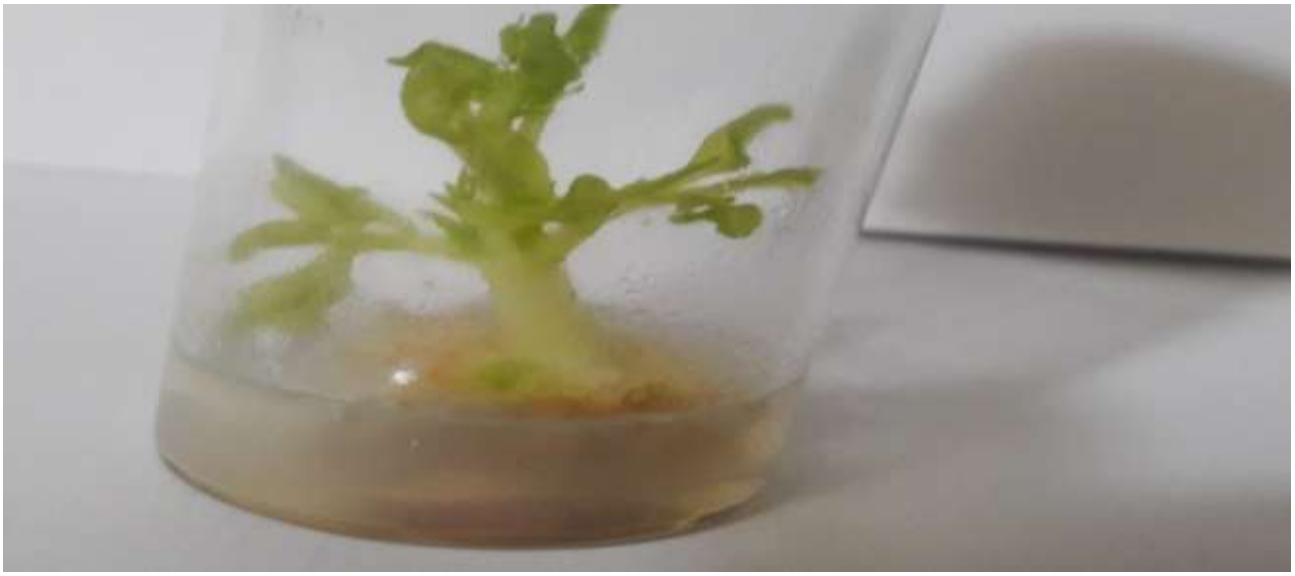

**Figure 2: Shoot proliferation from *M. oleifera* callus grown on MS medium supplemented with 1.0 mg.L$^{-1}$ Kin and 0.4 mg.L$^{-1}$ NAA after three months**

The present study showed that when Kin was augmented from 1.0 mg.L$^{-1}$ to 1.5mg.L$^{-1}$, higher shoot proliferation percentage was obtained. In general, low auxin and higher cytokinin levels are preferable for callus proliferation. This result is similar to the results of [18] who reported that MS medium containing high concentration of Kin combined with low concentration of IBA produced the highest shoot proliferation percentage of M. *oleifera* callus.

In contrast, [19] reported that MS medium containing low concentration of Kin with high concentration of IBA produced the highest shoot proliferation percentage of *M. oleifera* callus. The increase in mean % shoot proliferation may be due to that added growth regulators are effective enough on re differentiation of callus to organized tissues then proliferation [12]. The present study indicated that in case of indirect organogenesis, shoot proliferation from *M. oleifera callus* was found highly dependent on the level and type of growth regulators. All growth regulators exhibited shoot proliferation and varied in their potential and efficiency.

## Rooting

Production of plantlets with profuse roots *in vitro* is important for successful establishment of regenerated plantlets. Shoots were transferred onto MS medium supplemented with2.0 mg.L$^{-1}$ IBA for rooting. The rooting percentage was 100% for shoots regenerated from callus cultures (Figure 3).

These results are in agreement with those obtained by [11] in *M. oleifera* who reported that profuse roots were seen on *in vitro* regenerated shoots grown in MS medium supplemented with IBA. [20]. Reported that the highest number of M. *oleifera* roots per shoot occurred in MS medium containing IBA. Auxins promote root initiation by catalyzing cells to divide. Auxin also induces both pre-existing roots and adventitious root formation [21].

 

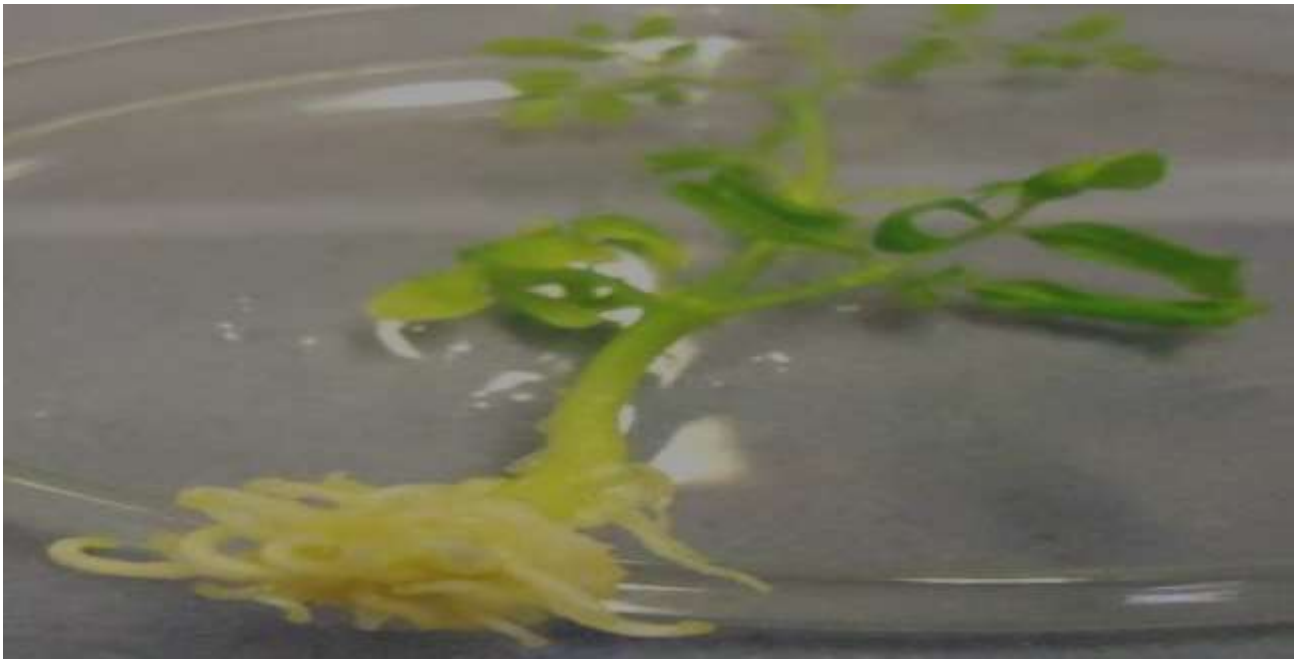

**Figure 3: Root formation on the bases of *M. oleifera* shoots regenerated from callus cultures transferred to MS medium supplemented with2.0 mg.L$^{-1}$ IBA after one month**

## Acclimatization of Plantlets

Out of 70 rooted shoots transplanted *ex vitro*, survival of 80% was achieved after one month of transfer to pots when shoots were covered with plastic bottles (Figure 4). All acclimatized plantlets were grown in pots containing 1:1mixture of soil and sand. A developmental period of six months was required for the production of plants of *M. oleifera* starting from surface sterilization stage to acclimatization. Similar result was reported by [25], in which 90% rooted shoots of *M. oleifera* were successfully transferred to 1:1 mixture of soil and sand. This high survival rate also was recorded in studies performed by [16], whereby a survival rate of more than 90% was observed in plantlets survival of M.oleifera.

**Figure 4: Acclimatization of *M. oleifera* plantlets, two weeks after transfer to pots inside a growth chamber and covered with plastic bottles**

## Conclusion

Results of this study indicate that large scale propagation of *M. oleifera* by tissue culture methods is feasible and several plantlets can be regenerated from a single stem and leaf explants.

 

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