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EFFECTS OF PLANT GROWTH REGULATORS ON THE CALLUS OF ROSE LEAVES

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Keywords: Rosa hybrida, Leaf, Plant growth regulator, Callus

Abstract

In the present study effects of different plant growth regulator combinations on leaf callus growth in two *Rosa hybrida* cultivars, namely *R. hybrida* 'Ingrid Bergman' and R. hybrida 'Xindongfang', were analyzed. The plant growth regulators were added into MS, MS + 2,4-D (1.5 mg/l, 2.5 mg/l, 3.5 mg/L, and 4.5 mg/l) and MS + 2.0 mg/l NAA + 1.0 mg/l ZT, MS + 2.0 mg/l NAA + 1.0 mg/l KT, MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA and MS + 2.0 mg/l NAA. The leaves were treated as broken and cultured on these nine different media types. Results showed significant differences in color, morphology, and callus quantity. In the first medium, the callus of rose leaves was the best on MS + 4.5 mg/l 2,4-D medium, followed by MS + 2.5 mg/l 2,4-D and MS + 3.5 mg/l 2,4-D, and finally MS + 1.5 mg/l 2,4-D. No callus was found in MS medium. In the second kind of medium, the leaves grow callus, and the callus grew best on MS + 2.0 mg/l NAA + 1.0 mg/l ZT medium, followed by MS + 2.0 mg/l NAA + 1.0 mg/l KT, finally MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA, MS + 2.0 mg/l NAA medium, the callus grew loose and dry, and lost luster after a few days. Therefore, 2,4-D, KT, ZT, 6-BA, and NAA can promote the formation of callus. With the increase of concentration of 2,4-D, the callus induction rate increased, and the color of the callus also changed significantly. The combination of KT, ZT, and NAA had an obvious effect on callus induction and expansion.

Introduction

Rosa hybrida L. is a semi-evergreen or perennial evergreen shrub belonging to the genus *Rosa* of Rosaceae is an essential woody ornamental flower widely cultivated all over the world. Apart from its ornamental value, it is also used for the production of essential oil and vitamin C and rightly called the queen of flowers.

Currently propagation of roses is typically done through prunning on commercial level. However other methods such as sprouting and grafting can also be used. Nevertheless all these approaches are laborious and time consuming. The rates of multiplication as well as influence of climatic factors are significant limitations to the conventional expansion. On the other hand tissue culture has become a popular alternative for rose propagation. Micropropagated plant have several other advantages. These plants are better suited for the production of cut flowers as they are more compact. Important features of *in vitro* expansion also include its excellent qualitative potential and the possibility of production of healthy and disease free plant all the year round.

Many factors influence the rose regeneration process (Chen *et al*. 2011, Salehi *et al*. 2017). There is a significant difference in regeneration ability among different cultivars of rose. Additionally, different types and concentrations of plant growth regulators added to the medium affect callus growth (Zurn *et al*. 2020, Xu *et al*. 2014). Considering the importance of this plant and to overcome the problem of propagation through conventional methods, attempt has been

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made to develop regeneration methods for clonal propagation (leaf callus) of this ornamental plant. In the present experiment, different materials such as *R. hybrida* cultivars 'Ingrid Bergman' and 'Xindongfang' were cultured in nine types of media with different combinations of plant growth regulators to observe the formation of leaf callus.

Materials and Methods

The experimental materials were two *Rosa hybrida* cultivars: *R. hybrida* 'Ingrid Bergman' and *R. hybrida* 'Xindongfang'. Tissue culture seedlings (20–25 days) of *Rosa hybrida* were used in the experiments. The callus was induced by MS medium (containing 20 g/l sucrose and 8.0 g/l agar, pH 5.8) combined with different ratios of 2.4-D, KT, ZT, 6-BA, and NAA. The callus was then autoclaved at 121°C for 25 min.

On the ultra-clean workbench, the top leaves of the rose without seedlings were cut off, and leaves of the same size (Fig. 1A) were selected and inoculated back down on the medium supplemented with different 2.4-D concentrations (Table 1) and different plant growth regulators (Table 2) for culture. Each medium was treated with six vials of 20 leaf plates or whole leaves. The culture conditions were as follows: temperature (25 ± 2°C), light intensity (8000-10000 lx), and photoperiod (16 hrs light/8 hrs dark).

The quantity, color, and texture of calli were observed regularly, every 3 days as an observation period.

The callus induction rate under different treatments was measured. A total of 20 explants per bottle were counted as one repeat, and there were six replicates in total. Callus induction rate = (number of explants forming callus/total number of explants inoculated) ×100%. SPSS 22.0 software was used for variance and correlation analysis.

Result and Discussion

When the concentration of 2,4-D was 4.5 mg/l, the callus incidence of 'Xindongfang' and 'Ingrid Bergman' was highest, 95.8% and 13.3%. When the concentration of 2,4-D was 1.5 mg/l, 2.5 mg/l, and 3.5 mg/l, the callus' color was pale yellow and relatively moist and compact, but when 2,4-D was 4.5 mg/l, the color of the callus turned pale yellow and white. The leaf disc of Chinese rose 'Ingrid Bergman' was inoculated on the medium, only a few of the main veins of the incision of the leaves grew callus, and the rest of the leaves turned yellow. When the concentration of 2,4-D was 1.5 and 2.5 mg/l, the callus' color was white and loose when the concentration of 2,4-D was 3.5 and 4.5 mg/l. The callus' color turned pale yellow. So the callus incidence increased with the addition of 2,4-D, and the number of calli increased gradually. In addition, 'Xindongfang' could induce the formation of moist and dense calli when the concentration of 2,4-D was low, but 'Ingrid Bergman' might need a higher concentration of 2,4-D to form a moist and dense callus (Table 1).

They leaves turned brown and yellow at the incision on the 3th day when they were inoculated on MS + 4.5 mg/l 2,4-D. On the 6th day, the browning of the incision was aggravated, and the leaf edge was curved inward. On the 9th day, the main veins of most incisions had calli, and 2/5 of the leaves had a pale yellow callus, which was moist and compact. On the 12th day, a light yellow callus appeared in half of the incision, and the callus was enlarged. By day 15, all the leaves in the bottle had grown pale yellow calli. On the 18th day, the callus' volume was more extensive than that of the previous callus, and the callus was moist and compact (Fig. 1). The growth of leaves in the first 6 days after inoculation on the medium of MS + 4.5 mg/l 2,4-D was similar to that of 'Xindongfang.' On the 9th day, the leaf yellowing was aggravated, and the incision area had

increased browning. On the 12th day, a small part of the leaves was yellowing. Only little leaves had callus induced from the cut of the principle vein of leaves (Fig. 2).

Table 1. Effects of different 2,4-D concentrations on the growth of leaf disc callus.

Culture medium	*R. Hybrida* 'Xindongfang'		*R. Hybrida* 'Ingrid Bergman'	
	Callus formation rate	Callus features	Callus formation rate	Callus features
MS	0.0 d	No callus	0.0 c	No callus
MS + 1.5 mg/l 2,4-D	83.3 c	Pale yellow, moist	9.2 ab	The main veins of the incision are long and white
MS + 2.5 mg/l 2,4-D	85.8 bc	Pale yellow, moist	6.7 abc	The main veins of the incision are long and white
MS + 3.5 mg/l 2,4-D	93.3 ab	Pale yellow, moist	4.2 bc	The main veins of the incision are long and pale yellow
MS+ 4.5 mg/l 2,4-D	95.8 a	Pale yellow and white, moist	13.3 a	The main veins of the incision are long and pale yellow

Means within the same line followed by the different small letters are significantly different at 5% level.

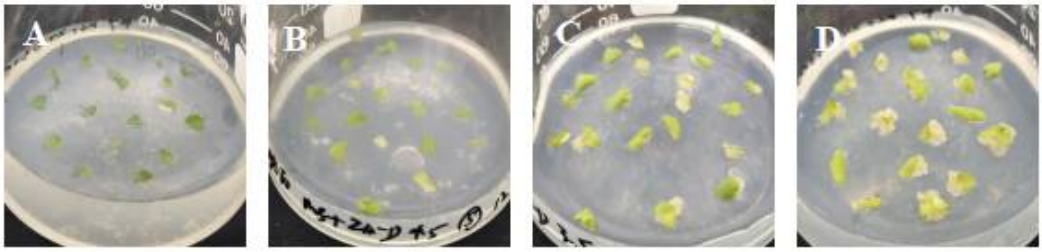

Fig. 1. Callus formation process of MS + 4.5 mg/l 2,4-D 'Xindongfang' leaf disc. A. leaves in day 1. B. leaves in day 6. C. leaves in day 12. D. leaves in day 18.

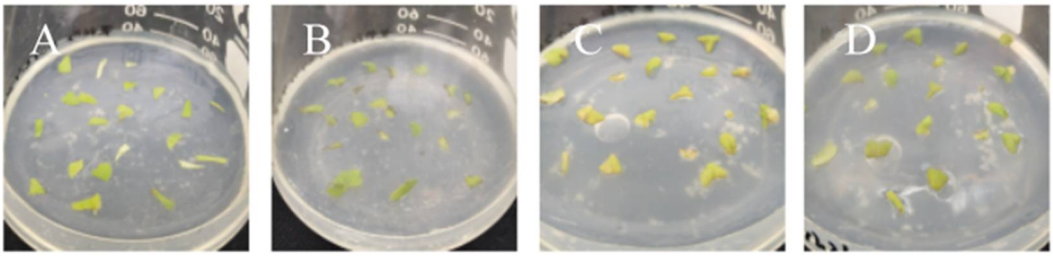

Fig. 2. Callus formation of MS + 4.5 mg/l 2,4-D 'Ingrid Bergman' leaf disc. A. day 1. B. day 6. C. day 12. D. day 18.

On the medium MS + 2.0 mg/l NAA + 1.0 mg/l ZT and MS + 2.0 mg/L NAA + 1.0 mg/l KT, the callus grown from the cut of the 'Xindongfang' leaves was fresh and green, wet and dense. The surface of callus was granular, and the number of callus was big. On the medium of MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA, the callus quantity was increased. On MS + 2.0 mg/l NAA medium, the callus' color was milky white, part of the callus was brown, dry, and loose, and the number of calli was small.

The first callus from the cut of 'Ingrid Bergman' leaves appeared on the medium of MS + 2.0 mg/l NAA + 1.0 mg/l ZT and MS + 2.0 mg/l NAA + 1.0 mg/l KT, followed by the medium of MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA. Leaf disklets of 'Ingrid Bergman' were inoculated with different plant growth regulators. The first full callus was on MS + 2.0 mg/l NAA + 1.0 mg/l ZT medium, followed by MS + 2.0 mg/l NAA + 1.0 mg/l KT medium, and finally in MS + 2.0 mg/L NAA + 1.0 mg/L 6-BA medium. Therefore, MS + 2.0 mg/l NAA + 1.0 mg/l ZT was the most suitable medium for callus induction of 'Xindongfang' and 'Ingrid Bergman' (Table 2).

Table 2. Effects of different combinations of plant growth regulators on the formation of leaf disc callus.

Culture medium	*R. Hybrida* 'Xindongfang'	*R. Hybrida* 'Ingrid Bergman'
MS + 2.0 mg/l NAA	Small amount, milky white, dry loose	Small amount, milky white, dry loose
MS + 2.0 mg/l NAA + 1.0 mg/l ZT	Large quantity, light green, moist, and compact	Large quantity, light green, moist, and compact
MS + 2.0 mg/l NAA + 1.0 mg/l KT	Large quantity, light green, moist, and compact	Large quantity, light green, moist, and compact
MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA	Large amount, light green, moist, and dense	Large amount, light green, moist, and dense

The incision leaves turned brown and yellow on MS + 2.0 mg/l NAA + 1.0 mg/l ZT medium on the 3th day. On the 6th day, the leaf margin curved inward. On the 9th day, half of the incisions showed a light blue callus, which was moist and compact. On the 12th day, all the incisions showed a light blue and enlarged callus. After the 15th day, the callus continued to expand, moist and compact, with a green texture, and the induction effect was good (Fig. 3). On the 6th day, after inoculation of leaf disks on MS + 2.0 mg/l NAA medium was similar to other leaves. On the 9th day, light yellow and milky white callus appeared in half of the incisions, which were dry and loose. On the 12th day, most calli appeared white, while some calli turned brown, dry, and loose (Fig. 4).

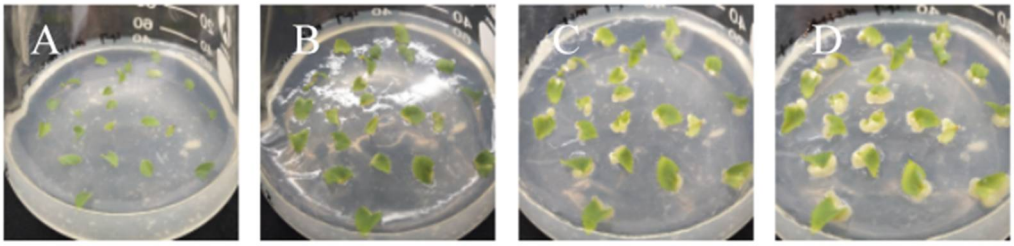

Fig. 3. The callus formation process of MS + 2.0 mg/l NAA + 1.0 mg/l ZT in the leaves of 'Xindongfang'. A. day 1. B. day 6. C. day 12. D. day 18.

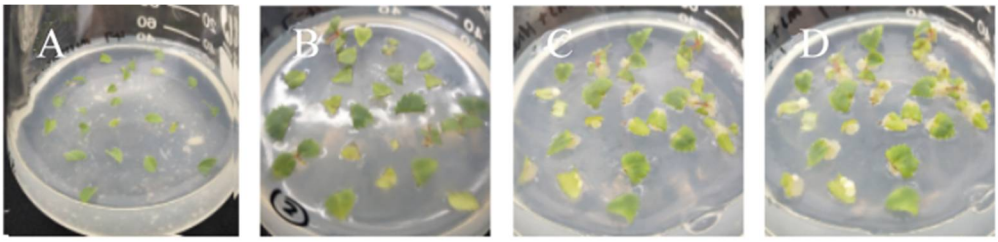

Fig. 4. The callus formation process of MS + 2.0 mg/l NAA'Xingdongfang' leaf disc. A. day 1. B. day 6. C. day 12. D. day 18.

The whole leaves of Chinese rose 'Xindongfang' inoculated on different 2,4-D concentrations could induce more calli to grow. The incidence of calli increased with the increase of 2,4-D concentration. When the concentration of 2,4-D was 4.5 mg/l, the callus incidence was as high as 96.7%, and the number of calli also increased gradually. The callus' color was light yellow, moist, and dense, and the texture was crisp. When the concentration of 2,4-D was 4.5 mg/l, the callus induction rate was 74.2%, but the callus number decreased. The color of the callus was light yellow, moist, and loose. Therefore, 'Xindongfang' induced moist and dense callus formation when the concentration of 2,4-D was low, but 'Ingrid Bergman' induced moist and loose callus formation when the concentration of 2,4-D was different and the callus amount was low. When the whole leaf was used as explants, the small incision influenced the callus' differentiation. There was some difference in callus growth between whole leaves and leaf plates of Chinese rose 'Xindongfang', but the growth pattern was similar to that of the leaf disc. However, the growth characteristics of calli from the whole leaves of 'Ingrid Bergman' showed significant changes (Table 3).

Table 3. Effects of different 2,4-D concentrations on the growth of whole leaf callus.

Culture medium	*R. hybrida*'Xindongfang'		*R. hybrida*'Ingrid Bergman'	
	Callus formation rate	Callus features	Callus formation rate	Callus features
MS	0.0 d	No callus	0.0 d	No callus
MS + 1.5 mg/l 2,4-D	40.3 c	Pale yellow, moist	50.8 c	Light yellow, moist, and loose
MS + 2.5 mg/l 2,4-D	71.7 b	Pale yellow, moist	60.0 b	Light yellow, moist, and loose
MS + 3.5 mg/l 2,4-D	91.7 a	Pale yellow, moist	67.5 a	Light yellow, moist, and loose
MS + 4.5 mg/l 2,4-D	96.7 a	Pale yellow, moist	74.2 a	Light yellow, moist, and loose

Note: means within the same line followed by the different small letters are significantly different at 5% level.

The whole leaves of Chinese rose cultivars 'Xindongfang' and 'Ingrid Bergman' were inoculated on the medium with different combinations of plant growth regulators. On the medium MS + 2.0 mg/l NAA + 1.0 mg/l ZT MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA and MS + 2.0 mg/l NAA + 1.0 mg/l KT, light green callus grew at the petiole, moist and compact, with clear texture, numerous callus, and fine granules on the surface. In the medium of MS + 2.0 mg/l NAA, the callus' color was light yellow, part of the callus was brown, dry, and loose, and the number of calli was small.

The whole leaves of Chinese rose 'Xindongfang' were inoculated on the medium with different combinations of plant growth regulators. On the 9th day, callus was induced from the petiole on the medium MS + 2.0 mg/l NAA + 1.0 mg/l ZT, MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA and MS + 2.0 mg/l NAA + 1.0 mg/l KT. On the 15th day, callus was induced from all the media. On the 9th day, callus grew on MS + 2.0 mg/l NAA + 1.0 mg/l ZT and MS + 2.0 mg/l NAA + 1.0 mg/l KT, on the 12th day, callus grew on MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA, and on the 15th day, callus grew on all the media. Therefore, MS + 2.0 mg/l NAA + 1.0 mg/l ZT and MS + 2.0 mg/l NAA + 1.0 mg/l KT could be suitable media for callus induction in 'Xindongfang' and 'Ingrid Bergman' (Table 4).

The whole leaves of 'Xindongfang' and 'Ingrid Bergman' were inoculated on MS + 4.5 mg/l 2,4-D. On the 3th day, the incision of the petiole turned brown. On the 6th day, the browning of the incision was aggravated, and the leaf edge was curved inward. On the 9th day, the incision of the petiole was enlarged. On the 12th day, 2/3 of the petiole incisions showed yellowish callus, wetting, and compacting. On the 15th day, some of the calli were loose, and the leaves were

yellow. On the 18th day, the callus volume was higher than that of the previous callus, and the callus was enlarged, moist, and loose, and the leaves were wilted (Fig. 5).

Table 4. Effects of different combinations of plant growth regulators on the growth of whole leaf callus.

Culture medium	*R. hybrida* 'Xindongfang'	*R .hybrida* 'Ingrid Bergman'
MS + 2.0 mg/l NAA	Large volume, light yellow, moist, and compact	Large volume, light yellow, moist, and compact
MS + 2.0 mg/l NAA + 1.0 mg/l ZT	Large quantity, light green, moist, and compact	Large quantity, light green, moist, and compact
MS + 2.0 mg/l NAA + 1.0 mg/l KT	Large quantity, light green, moist, and compact	Large quantity, light green, moist, and compact
MS + 2.0 mg/l NAA + 1.0 mg/l 6-BA	Large quantity, light green, moist, and compact	Large amount, light green, moist, and dense

The whole leaves of rose cultivars 'Xindongfang' and 'Ingrid Bergman' were inoculated on the medium of MS + 2.0 mg/l NAA and 1.0 mg/l ZT, and the growth rate in the first 9 days was consistent with that of MS + 2,4-D 4.5 mg/l. On the 12th day, 2/3 of the petiole incisions showed yellowish callus, wetting, and compacting, and on the 15th day, all petioles had pale green callus, moist and compact. On the 18th day, the callus increased, expanded, and became moist and compact (Fig. 6).

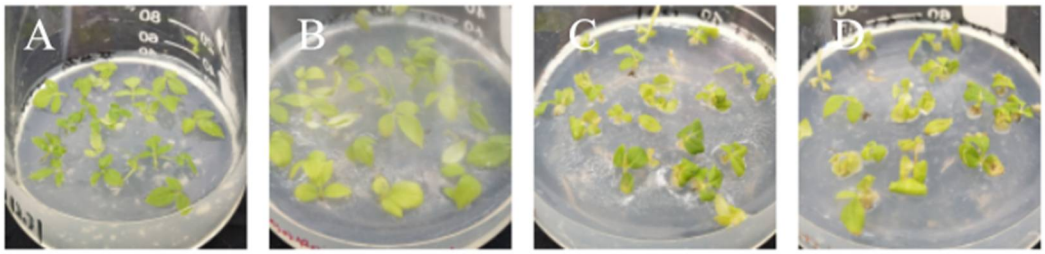

Fig. 5 Callus formation of MS + 4.5mg/l 2,4-D 'Ingrid Bergman' whole leaves. A. day 1. B. day 6. C. day 12. D. day 18.

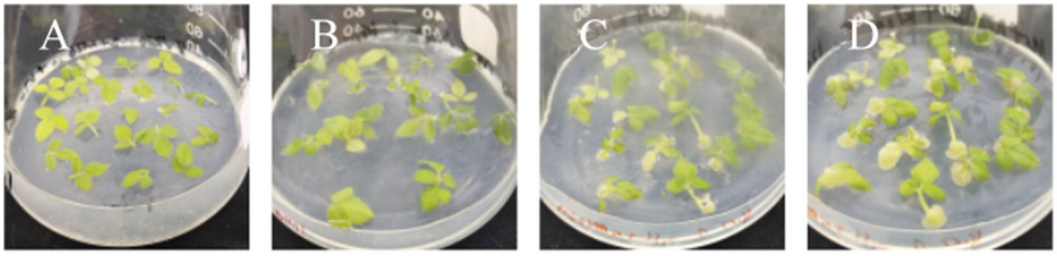

Fig. 6. The callus formation of MS + 2.0mg/L NAA + 1.0 mg/l ZT 'Ingrid Bergman' whole leaves. A. day 1. B. day 6. C. day 12. D. day 18.

Results of the present study showed that growth regulators such as 2,4-D, NAA, KT, ZT, and 6-BA promoted the formation of a rose callus (Kamo *et al.*2004, Valizadeh *et al.* 2009, Yi *et al.* 2014). The callus formed with only 2,4-D added was pale yellow in color and compact in texture. With increasing concentrations of 2,4-D, the callus quantity increased significantly, and the color

also changed. The callus treated with NAA alone was pale yellow or white, but some turned brown with dry and loose texture over time. However, after the addition of NAA, KT, ZT, or 6-BA, the callus turned pale green, had a clear texture, and was moist and compact (Theo *et al.* 1996, Liu *et al*. 2017). Tang *et al*. (2017) found that 2,4-D, NAA, KT, ZT, and 6-BA are usually used as the key hormones for plant callus formation. In the process of callus formation, adding a certain concentration of 2,4-D or NAA allowed for easier callus formation compared to cytokine alone. Pan (2011) found that KT, ZT, and 6-BA are required in the culture medium for proper callus regeneration of *Rosa hybrida*, and a certain proportion of plant growth regulator combinations had a significant effect on callus induction. Feng *et al*. (2014) showed that 6-BA, as a cytokinin, could promote the formation of most plant calli at specific concentrations, and when the concentration of 2,4-D was added to the medium at a certain concentration, the degree of callus browning was high, which had a negative effect on callus regeneration. It may be concluded that obtained results are consistent with that of the MS control treatment (without any growth-regulating substance), where there was no callus formation in the leaves. However, there were significant differences in callus growth between whole leaves and crushed leaves on MS+2.0 mg/l NAA medium, in addition, the addition of KT, NAA, ZT, and 6-BA with different concentrations and proportions was better for callus formation than 2,4-D.

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