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More about *in vitro* grape virus symptomatology

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Summary. An *in vitro* grafting method adapting the usual "indexing by green grafting" technique (Walter *et al.*, 1990) to *in vitro* culture conditions was tested. The local grapevine varieties Khamri Tozeur, Akhel Meguergueb, Jerbi Degueche, Asli and Jebbi, infected respectively with infectious degeneration, leafroll, vein mosaic, corky bark and vein necrosis diseases, were used. Virus expression was greater on media having a greater number of nutrients such as the Van Hoof (1974) medium containing 12 macronutrients. On the other hand, the addition of BAP (0.25 mg l^{-1}) to the medium reduced external virus symptoms on newly sprouted axillary shoots. When these shoots were transferred to fresh culture medium supplemented with IBA (0.1 mg l^{-1}), typical and specific symptoms of major virus diseases clearly developed. Re-grafting of axillary shoots on the fragment of an infested clone can be used to overcome difficulties related to corky bark and vein mosaic symptom expression. We also demonstrated that viruses occur in general in mixed infections. The symptoms of a given virus become evident only when favourable conditions to it arise. Our research is still working on reducing the time of detecting virus and virus-like diseases. This is essential for sanitary selection of grapevine plants.

Key words: BAP, Tunisian vines varieties, nutrient variability, symptoms, virus association.

Introduction

Viral diseases in Tunisian vineyards fall into 2 groups:

1. diseases caused by viruses that have been clearly identified such as *grapevine fanleaf virus* (GFLV) and *grapevine leafroll associated viruses* (GLR-aVs);
2. virus-like diseases of unknown etiology, such as vein necrosis and vein mosaic.

Viruses of the former group can be detected by immunological techniques such as ELISA. For viruses in the latter group clones have to be grafted onto indexing varieties (indicator vines) by means of a green grafting technique (Walter *et al.,* 1990). Despite its efficiency in revealing virus symptoms in a short time, this technique may require elaborate equipment (highly regulated and air-conditioned greenhouses). However, the needs of grape growers are pressing.

The aim of this work was to study the virus symptomatology of *in vitro*-grown grapevine, and also to shorten the time for detection of viruses and virus-like disease symptoms.

Materials and methods

Plant material

Plant material consisted of 3 herbaceous cutting plots grown respectively with:

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1. indicator varieties *(Vitis rupestris,* Cabernet Sauvignon, Riparia Gloire, Kober 55, 110 R*)* grown under greenhouse conditions, where their sanitary status was periodically checked.
2. local varieties (Khamri Tozeur, Akhel Meguergueb, Jerbi Degueche, Asli, Jebbi) exhibiting symptoms of virus diseases (respectively infectious degeneration, leafroll, vein mosaic, corky bark, vein necrosis) used as clones to be tested
3. the same local varieties regenerated from meristematic culture, and which tested negative by serological means were used as controls.

In vitro culture

Herbaceous cuttings from the indicator varieties and clones symptomatic were externally disinfected separately in a 9% calcium hypochlorite solution with some drops of Tween 20.

A fragment of each clone was grafted on cuttings of an indicator variety with an axillary bud. The graft zone was covered with aluminium foil.

In order to study the effect of the culture medium on virus expression, four culture media were tested: diluted Murashige and Skoog's medium (1962) MS/2; MS/2 supplemented with 6-benzylaminopurine BAP (0.25 mg l^{-1}); Van Hoof (1974); and Van Hoof supplemented with BAP (0.25 mg l^{-1}). For each medium, 30 grafts infected at least by fan-leaf or leafroll were tested. Statistical analysis was performed with the STATISTICA package (Statistical Analysis System, Version 5, Cary, NC, USA) using Analysis of variance (ANOVA) procedure with the Tukey test.

In these conditions, virus symptoms are expected to appear on the indexing bud. When symptoms were not clear, shoots that sprouted from the indexing bud were cultured on Van Hoof medium supplemented with indol-butyrric acid (IBA) (0.1 mg l^{-1}).

In order to confirm a diagnosis, a second *in vitro* grafting method was applied, in which the indicator variety and the test clone were grown in a tube, and microcuttings taken from shoots of the indicator varieties were grafted on an internode of the test clone.

Serological tests

Samples of different sizes were checked for GFLV and GLR-aVs viruses. ELISA test, to screen and identify the viruses were carried out according to Clark and Adams (1977). Sample extracts from grapevines were crushed in a standard buffer solution and tested by the DAS-ELISA sandwich method (Cambra *et al.,* 1991). The serum used was provided by Bioreba (Reinach, CH). Preliminary results were recorded visually, samples were then transmitted to a Multiskan with an optic density of 405 nm.

Results and discussion

Effect of cytokinins addition and nutrient variability

Growth of control combinations on MS/2 medium was normal. Shoots growing from the index buds of the indicator varieties showed vigorous aerial parts and rooting systems without any virus symptoms. This confirmed that plants regenerated through meristem culture were healthy (Ben Abdallah, 1999).

Although the index bud burst of the tested combinations was normal, a deterioration of most grafts was noted at the end of culture. This deterioration was often followed by the death of major grafts. The few saved grafts lacked a rooting system and aerial parts allowing the display of visible symptoms. These results were similar to those of Abracheva *et al.* (1994) on *Vitis rupestris* with infectious degeneration and corky bark and seemed to indicate that a virus was the cause.

For all control combinations, serological tests were negative, while the tests on deteriorated grafts showed that such grafts were affected at least by infectious degeneration or leafroll. This suggests that the death of grafts following their deterioration was due to physiological alterations induced by pathogenic agents.

In order to obtain long shoots with virus symptoms on the aerial parts, while yet avoiding shoot deterioration, it was necessary to find a suitable growth medium for the test combinations.

MS/2 hormone-free medium was found to produce fewer grafts with virus symptoms than the Van Hoof medium. With both hormone-free media, however, there was a substantial number of deteriorated grafts, ranging between 57 and 88% (Fig. 1). When BAP was added (0.25 mg l^{-1}), there was a high proportion of grafts with virus symptoms in both media, but it was significantly greater in the Van Hoof medium.

On the other hand, symptoms were observed

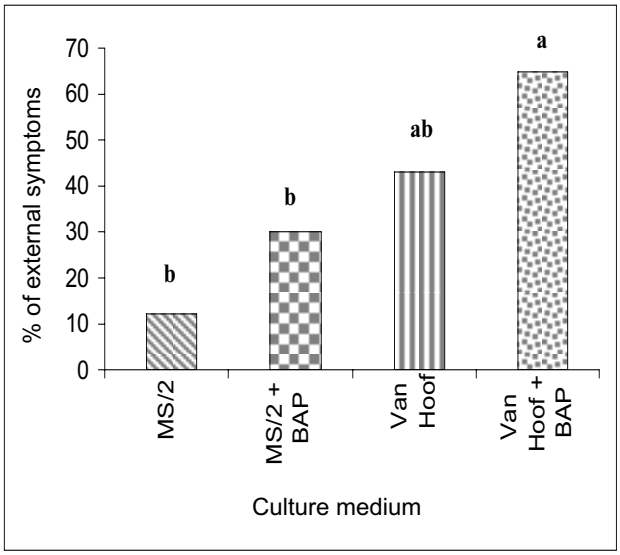

Fig. 1. Effect of nutrient variations and BAP addition on grape virus expression. Means with different letters show significant difference at $P \leq 0.01$ using Tukey test.

clearly when shoots that sprouted from indexing buds were immediately transferred to Van Hoof medium with IBA. The Van Hoof medium therefore stimulated virus expression more than MS/2 medium, with or without BAP (Fig. 1). This may be attributed to the fact that the appearance of virus symptoms is favoured by the greater number of nutrients provided by this medium (12 macronutrients).

Our results were consistent with Hassani and Boubals (1991) who reported that virus expression was enhanced in a culture medium with many nutrients. Moreover, cytokinin addition also seems to favour virus expression on new shoots sprouting from the index bud. This suggests that cytokinins provide favourable conditions and more chances for latent viruses to be expressed by stimulating cellular proliferation of axillary meristems, and by inducing new shoot development.

Virus symptomatology

Generally, axillary shoots from the indexing bud grew better than scions. Scion growth always remained limited, indicating the formation of vascular connexions between scion and rootstock. Virus transmission is thought not to be related to callus formation in the grafting zone (Ben Abdallah, 1999).

Vein necrosis

Three weeks after the Jebbi variety was grafted on 110 R rootstock, black necrotic spots appeared along the basal leaf veins of 110 R. These spots gradually spread along the leaf veins and developed into extended black necrosis of all veins and veinlets (Fig. 2). If a temperature of 27°C and lighting of 4000 lux were maintained, symptoms of this virus appeared consistently whenever an infected clone of *Vitis vinifera* was grafted on 110 R rootstock regardless of the type of medium used.

The symptoms of vein necrosis generally appeared before shoot deterioration. Shoot transfer to another medium was not required. Symptoms of this disease were evident even when hormone-free culture media were used. Dkhili and Grenan (1995) succeeded in producing symptoms of vein necrosis on the leaves of 110 R rootstock cultivated *in vitro*.

Leafroll

A few days after Cabernet Sauvignon axillary shoots developed on Van Hoof (1974) medium with BAP, scattered red spots were observed on the inter-vein spaces of the first leaves of these shoots (Fig. 3).

By transferring these shoots to Van Hoof (1974) medium with IBA (0.1 mg l^{-1}), these spots, representing early leafroll symptoms (Dumont *et al.,* 1992), overran the entire leaf surface. The interveinal areas turned reddish. The specific symptoms of leafroll, such as downward rolling of the leaf underside, were observed in 6 weeks indicating that "Akhel Meguergueb" was infected with leafroll.

Infectious degeneration

Shoot basal leaves exhibited slower growth. This was followed by mild to severe atrophy. With most combinations, infectious degeneration symptoms on the newly developing shoots were unclear. However, when shoots were transferred before degeneration to MS/2 (1962) or to Van Hoof with IBA, the specific symptoms of infectious degeneration were observed clearly along the aerial parts after 30 days. These symptoms were:

- asymmetry and deformation of the leaf blade with loss of grape leaf characteristics (Fig. 4);
- flat and forked shoots (Fig. 5);
- shortening of internodes.

Vein mosaic

Although symptoms of vein mosaic appeared on "Jerbi Degueche" leaves before grafting, they were difficult to distinguish on the new shoots of the index bud. They were randomly detected on Riparia Gloire axillary shoots. Symptoms of vein mosaic were not very clear on shoots grown on Van Hoof medium with IBA. However, symptoms were clear if Riparia Gloire axillary shoots were re-grafted on an internode of Jerbi Degueche (the clone to be tested). Symptoms consisted of a pale green discoloration along the main vein and veinlets, giving the blade a mosaic aspect.

Corky bark

Most grafts on Kober 55 deteriorated before they could develop. For those grafts that did survive, no symptoms appeared on the axillary shoots of indicator varieties even when they were transferred to Van Hoof medium with IBA. However, when microcuttings from these axillary shoots were re-grafted on an internode of the clone to be tested, corky bark symptoms similar to those described by Dumont *et al*. (1992) were observed after 2 months. These consisted of outward bark browning followed by bark detachment (Fig. 6). The death of the whole plant scion followed. These findings were similar to those of Tanne *et al*. (1993), who found corky bark symptoms *in vitro* on the herbaceous stem of the rootstock.

These findings as well as those on vein mosaic and infectious degeneration confirm that grapevine plants can be virus-infected without showing visible symptoms. Such plants, in which the virus is latent are known as healthy carriers (Walter, 1988).

Evidence of virus association

In the experimental conditions, many grapevine clones with symptoms of the viral diseases tested for showed other virus symptoms as well. A particularly interesting and surprising finding was that of a clone with vein mosaic (Jerbi Degueche): when this clone was grafted on shoots growing from a Riparia Gloire index bud, it produced the symptoms of infectious degeneration, such as flat and forked shoots (Fig. 5). To our knowledge, the case of a clone infected with one virus that, when grafted, produces the symptoms of another virus has not been observed *in vitro* before. This is true for Cabernet Sauvignon, which is a known indicator variety of leafroll, but which also exhibits the asymmetry and basal leaf atrophy typical of infectious degeneration (Fig. 7).

This was further confirmed by the symptoms for vein necrosis observed on *Vitis rupestris* when it was expected that infectious degeneration symptoms would appear like those on the clone to be tested. These findings are inconsistent with Dkhili and Grenan (1995), who stated that such cases of symptoms-change did not occur on varieties of *Vitis vinifera*.

It is concluded that a given virus is often associated with other viruses, all in a latent state. When the conditions for a virus become favourable, its specific symptoms are expressed, while the others may remain latent. Serological experiments carried out by Walter (1985) on grapevine leafroll disease suggested that one viral disease may be brought about by many viruses. According to Hassani and Boubals (1991), the appearance of vein necrosis symptoms *in vitro* is probably the result of a reaction between pathogenic agents in *Vitis vinifera* clones and the vein necrosis virus infecting 110 R. Our findings are consistent with Szychwski *et al*. (1995) who stated that vein banding disease syndrome probably resulted from a synergistic interaction between viroids and the infectious degeneration virus.

Conclusions

Our results confirm that, when done correctly, the *in vitro* grafting method described here facilitates the study of grape viral disease symptoms by detecting major viruses more quickly. Symptoms appeared in less than 1 month for infectious degeneration, in 2 months for leafroll, and in 3 months for vein diseases.

Apart from vein necrosis virus, the grafts were already deteriorated before any appearance of symptoms. Most virus symptoms were hard to observe when grafts were grown on the usual hormone-free culture media. However, on culture media with a great variability of nutrients, viruses could be expressed on the shoots sprouted from the index bud.

On the other hand, the addition of cytokinins such as BAP seemed to stimulate virus symptom expression. By transferring axillary shoots to fresh culture medium supplemented with IBA, it was

Fig. 2. Symptom of vein necrosis virus as black necrosis along the veins and veinlets of 110 R leaves.

Fig. 3. Scattered red spots on Cabernet Sauvignon leaf blade as early symptom of leafroll.

Fig. 4. Loss of grape leaf characteristics (symptom of infectious degeneration).

Fig. 5. Flat and forked axillary shoots of Riparia Gloire as specific and typical symptoms of infectious degeneration.

Fig. 6. Outward bark browning and blackening of Kober 55 as symptoms of corky bark.

Fig. 7. Asymmetry and basal leaf atrophy exhibited on Cabernet Sauvignon shoots as symptoms of infectious degeneration.

possible to bring out clearly the typical and specific symptoms of the major viruses. Re-grafting the shoots to an internode of an infested clone is a method that can be used to overcome difficulties relating to corky bark and vein mosaic virus expression.

Our results suggest that viruses generally occur in mixed infections. The symptoms of a given virus will be expressed only when it encounters favourable conditions. The other viruses remain in a latent state. In addition, it appears that one virus may stimulate expression of the symptoms of other viruses.

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