Nutrient Medium Developed for the in vitro Demonstration of Sexual Reproduction by *Cryphonectriaparasitica*

Steven Jakobi
Amy Werner
Physical and Life Sciences Department
Alfred State College
Alfred, NY 14802
USA

Mark Double
Division of Plant and Soil Sciences
West Virginia University
Morgantown, WV 26506
USA

Abstract

Eighteen agar-based nutrient media were evaluated for their ability to support sexual reproduction (perithecia and viable ascospore formation) by the chestnut blight fungus, Cryphonectriaparasitica. Preliminary screening of each medium involved pairings of four sexually compatible isolates. If a medium appeared to support ascospore production, conidia (asexual spores) of 10 sexually compatible isolates of the pathogen were paired in 29 different combinations. Isolates were either recipients of conidia from a sexually compatible strain, or acted in reverse as the "male" donor of conidia. The most successful preparation was Leonian agar amended with 1 percent "light" coconut milk. Twenty-five of 29 pairings yielded ascopores in as few as 13 d (average 29 d) after establishment of crosses. Four isolates failed to develop ascospores when they were recipients of sexually compatible conidia, but were successful as spermatizing agents. These isolates most likely exhibited female sterility.

Keywords: Ascomycetes, chestnut blight, gender sterility, mating type, nutrient media, perithecia, sexual reproduction

1. Introduction

The chestnut blight fungus, *Cryphonectriaparasitica* (Murr.)Barr.), was accidentally introduced into North America at the turn of the 20th century from Asia (Kuhlman 1978, Milgroom et al. 1996). The subsequent spread of the fungus throughout the natural range of the American chestnut, *Castaneadentata*, was paralleled by increases in genetic diversity in populations of the pathogen (MacDonald and Double 1978, Milgroom and Cortesi 1999). The flow of genetic information between/among different populations of this fungus depends on two factors: vegetative compatibility (VC), which determines whether hyphae of two strains can fuse together to form a heterokaryon, and sexual compatibility, in which the union of two opposite mating types leads to karyogamy and formation of sexual spores (ascospores). Since sexual reproduction results in potentially significant genetic recombination, it is useful to determine the distribution of both VC types and mating types in the pathogen population. Virulent strains of *C. parasitica* induce the formation of lethal cankers in bark wounds of their susceptible hosts (Rankin 1914, Bramble 1936). While it is not known how much inoculum (mycelium or spores) is required for infection and disease progression (Kenaley et al. 2014), it is common to find several VC types in a single canker (Kuhlman 1982). When North American populations of *C. parasitica* are sampled, large numbers of VC groups are typically found in a given stand of chestnut stump sprouts. For example, a study in the Great Smoky Mountains National Park identified 52 VC groups among 159 blight cankers sampled (Double et al. 2014).

An additional complicating factor in examining the population structure of this fungus is that two vegetatively incompatible isolates may be sexually compatible if conidia rather than hyphae are the sources of union (Kuhlman and Bhattacharyya 1984, Marra and Milgroom 2001). The availability of a rapid and inexpensive method to determine the distribution of mating types is critical to understanding the population structure and evolution of this pathogen. The standard medium in most labs for growing in vitro cultures of *C. parasitica* is potato dextrose agar (PDA). Under normal growing conditions (25 C) mycelium of pathogenic strains of the fungus will fill a petri dish in about 4-7 d on this medium; conidia production (with or without light) in older parts of the thallus typically commences within 1-2wk after initiation of hyphal growth (Hindal 1982). Virulent forms of the pathogen produce yellow-orange mycelium. Conidia are produced in orange pycnidia with clearly discernible cirrhi and may be clustered or scattered in the mycelium. PDA will not facilitate ascospore production even when two vegetatively and sexually compatible isolates are grown together. Amendments to PDA (e.g. fruit extract, specifically strawberries) greatly enhance conidia formation (Marshall and Double 2014), but do not elicit the production of perithecia and ascospores. The most widely used current technique for determination of sexual compatibility between two strains involves co-inoculation into dormant American chestnut stems or bark pieces (Anagnostakis 1979; Willey 1980). Mycelium and conidia are first generated on PDA over a 1-2 wk period. Conidia are collected and co-introduced into autoclaved dormant chestnut stems or bark pieces to induce ascospore production. This method may produce observable perithecial necks in less than 30 d (Anagnostakis 1979), but frequently encompasses a much longer time frame (up to 3 months) for ascospore development (Jakobi 2005, Sun et al. 2009). The technique also requires suitably-sized dormant chestnut stems or bark pieces. While such material may be relatively easy to obtain in the eastern U.S., researchers in many parts of the world do not have ready access tosuch material. The development of an artificial medium to support the production of *C. parasitica* perithecia and ascospores has been an elusive goal, since PDA and other commonly used media only support lush mycelial growth and conidiation. The objective of this study was to find an inexpensive, readily available and rapid in vitro technique to induce ascospore formation by *C. parasitica.*

2. Methods and Materials

2.1. CryphonectriaParasitica Isolates

Isolates of known mating types were obtained from the West Virginia University culture collection (Table I). Since mating compatibility in this fungus is governed by a single gene and its two allelic forms, and the pathogen is, in most cases, dimictic (Burnett 1968, Anagnostakis 1994, McGuire et al. 2004), mating type 1 (Mat1; sometimes designated as "A" or "Mat 1-1") and Mat2 ("a" or "Mat 1-2") isolates were paired in various combinations. Initially, each candidate medium was evaluated by crossing four sexually compatible isolates (Mat1 "Schomberg" and "EP146" with the Mat2 isolates "EP155" or "Bockenhauer") in an attempt to generate perithecia with ascospores.

2.2 Media Selection

Candidate media for the production of sexual spores by C. parasitica were chosen from the literature (Tuite 1969, Difco Manual 1974) based on the success of the media in eliciting ascospore formation by other Ascomycetes. Eighteen media formulations were evaluated in this experiment (Table II). If a medium appeared to be at least partially successful, then additional mating crosses were set up (Table III). Each isolate was initially plated onto PDA (39 gm Difco PDA dissolved in 1000 ml distilled water and autoclaved for 20 min at 121 C, 15 psi; disposable petri plates filled with 25 ml PDA) and allowed to colonize the medium for 4-6 d at room temperature (20-25 C). No special lighting regimen was employed during the incubation. These cultures served as the inoculum source for the study of the experimental media. Test media were prepared and autoclaved using the same procedure as indicated for PDA. An amendment, such as hemp oil or coconut milk was added when an autoclaved medium cooled to about 50 C prior to pouring the plates. Small (2x2 mm) squares of each isolate were cut out of the PDA plates with a sterile scalpel and transferred to individual plates of the experimental media. Mycelial growth and asexual sporulation were assessed 5-7 d after inoculation. Conidial counts were initially performed by scraping the mycelium on one-half of the plate with a sterile transfer tool and immediately collecting a sample in 1 ml distilled water. Conidial counts were estimated with a hemacytometer. When pycnidia covered each plate, sexually compatible isolates were mated. One isolate served as the "female" and was spermatized by sexually compatible "male" spores. In most crosses the procedure was reversed, so that a plate of the former "male" donor now served as the "female" recipient of conidia (Table III).

Conidia for spermatization were obtained by flooding the surface of the sporulating culture with 5 ml 0.1 percent peptone water (1 g peptone dissolved in 1000 ml water and autoclaved). The surface of the colony was then gently scraped with a sterile wooden tongue depressor. Hyphae containing an average of 5 x 10^5 conidia/ml, were then removed with a sterile plastic pipet and 1 ml liquid was distributed on the surface of the recipient plate. After gentle agitation for 1 min to distribute the conidia evenly throughout the thallus of the recipient, the liquid was decanted and the plates were sealed with Parafilm. Treatments were replicated with a minimum of 4 or 5 plates. Cultures were evaluated with the aid of a stereoscopic microscope at 2-3 d intervals for development of clearly identifiable perithecial necks. A scale of 0-3 (0= no perithecia to 3= more than 15 perithecia/plate) was used to assess the degree of sexual sporulation by counting discernible perithecial necks on each replicate plate and averaging these numbers for the entire cross. When sexual reproduction was evident, several perithecia from randomly selected crosses were teased from the agar with a sterile dissecting needle to verify the presence of viable ascospores. Individual perithecia were crushed on a clean watch glass and asci were harvested. Ascospores were collected in 0.1 percent peptone water in a microcentrifuge tube, vortexed for 90 s and spread onto glucose yeast extract (GYE) medium (Lily and Barnett 1951), amended with 100 mg chlorotetracycline and 10 mg streptomycin sulfate. Ascospores were distributed evenly with a sterile glass rod and incubated for 24 hr at 83-86 C. Germinatingascospores were aseptically transferred to PDA plates and allowed to grow for 5-7 d for morphologic evaluation.

3. Results

Of the 18 different media formulation evaluated in this study (Table II), four were able to elicit the development of at least some perithecia. PDA alone, or PDA amended with vitamins, has never been shown to support sexual reproduction by *C. parasitica.* However, PDA amended with the liquid of boiled chestnut bark, Leonian agar alone, Leonian agar supplemented with vitamins, and Leonian amended with "light" coconut milk at least partially supported production of perithecia and ascospores

3.1PDA Prepared with Chestnut Bark Extract

Chestnut bark extract was used in this study to determine whether the chemical components of the bark can be used to supplement laboratory media to induce sexual reproduction by the pathogen. When PDA was prepared with chestnut bark extract (500-600 g chestnut bark boiled in 1500 ml distilled water for 0.5 h) instead of distilled water, crosses in 18 of 52 plates led to the formation of perithecia. The most successful cross (8 of 9 plates) involved EP155 as the recipient of EP146 "male" conidia. Perithecia were evident in as little as 14 d after incubation (2 plates), but the majority of crosses required 40 d before slender, black perithecial necks were visible. When the cross was reversed, (i.e. EP146 x EP155) the success rate was lower (3 of 10 plates) and required 35-40 d for evidence of sexual reproduction. When EP155 was the recipient of Schomberg conidia, 8 of 10 plates yielded perithecia in 15-35 d. The reverse cross (Schomberg x EP155) was unsuccessful even after 60 d incubation.

3.2 Leonian Agar without Amendments

In the Leonian agar (dihydrogen potassium phosphate 1.25 g; magnesium sulfate 0.625 g, peptone 0.625 g; maltose 6.25 g; malt extract 6.25 g in 1000 ml distilled water; Tuite, 1969) experiment 19 of 53 plates yielded perithecia. The most successful cross was EP155 x EP146 (12 of 16 plates; first perithecia seen after 23 d). EP146 x EP155 yielded fewer positive interactions (4 of 10 plates)and the earliest observable perithecial necks were seen at 28 d. Two of 5 Bockenhauer x Schomberg crosses produced perithecia (27 d) but 5 plates of Schomberg x Bockenhauer were negative. Although limited in success, the Leonian agar medium appeared to be a candidate for further experimentation.

3.3 Leonian Agar Amended with Thiamin and Biotin

Leonian agar amended with 10^{-2} percent thiamin and 10^{-2} percent biotin also was partially successful. Crosses in 23 of 40 plates resulted in formation of perithecia, but Schomberg x EP155, Schomberg x Bockenhauer, and EP146 x Bockenhauer failed to produce sexual spores. Leonian amended with 10^{-2} percent hemp oil appeared to be inhibitory for sexual reproduction.

3.4 Leonian Agar Amended with Coconut Milk

Because coconut milk is rich in protein, fatty acids, minerals and other nutrients (USDA 2014), it was chosen as an amendment to the Leonian agar. Concentrated coconut milk is produced by crushing or grating the flesh of fresh coconuts and then soaking the pulp for an extended period in water. This material is very thick and, due to its high saturated fat content, difficult to dissolve and disperse uniformly in aqueous media. Therefore, concentrated coconut milk was substituted by dilute canned coconut milk. Commercial brands of coconut milk, 55-60 percent diluted, were purchased in grocery stores and added to the Leonian medium at 1 percent concentration. This preparation was the most successful in vitro medium to support ascospore production by *C. parasitica*. Twenty-five of 29 crosses (86 percent) attempted with sexually compatible isolates resulted in production of ascospores (Table III). Eighty-one of 143 plates (56.7 percent) yielded at least some perithecia. Visible perithecial necks were observed in some cultures in as few as 13 d after spermatization, but some crosses required as many as 47 d to produce perithecia (Table III). The average number of days for appearance of perithecia in all crosses was 29 d, but some crosses produced perithecia on average in 18 d (EP146 x EP155), while others took an average of 31 d (MN-1 x Schomberg) or 40 d (6-3-1C x EP155). Some crosses did not yield perithecia on Leonian-coconut milk plates or on any other medium. For example, when Schomberg was the recipient of Bockenhauer conidia, there were no perithecia. However, if the Bockenhauer isolate was spermatized by Schomberg conidia, 3 of 5 replicates had perithecia. A similar situation was observed when Schomberg was the recipient of EP155 conidia (0 of 5 plates), while EP155 x Schomberg produced perithecia in all 7 Leonian-coconut milk plates.

4. Discussion

The Leonian agar medium amended with 1 percent light coconut milk was successful in inducing perithecia and viable ascospore production among 25 of 29 pairings involving 10 sexually compatible isolates of *C. parasitica*. The technique generally yields results more rapidly than the chestnut stem inoculation method, does not require the availability of bark of stem pieces from dormant American chestnut trees, and also may be applicable for demonstrating sexual reproduction or determining mating types in other Ascomycetes. The Leonian medium is made from readily available laboratory chemicals, supplemented by inexpensive cans of light coconut milk which can be purchased in the Asian foods section of most large grocery stores. Since one of the goals of this experiment was to find a convenient and rapid method to facilitate sexual sporulation, no special cultural conditions (e.g. climate-controlled room, specialized lighting for light/dark conditions) were used during the incubation period. One difficulty encountered in removing perithecia from an agar-based medium was that the agar matrix is rubbery and perithecia tended to slide or sink into the soft medium. This problem was overcome by removing and discarding the mycelium from around the perithecium, followed by spearing the perithecium with the point of a dissecting needle.

The percentage of crosses in which two strains of the fungus form sexual union, or the number of perithecia per cross may be improved, or the process speeded up in future studies under more exacting light and temperature conditions. Additional experiments with varying concentrations of light coconut milk added to the Leonian medium also may improve the results.

With respect to the failure of some sexually compatible crosses to produce perithecia, gender sterility among fungi is a widely recognized phenomenon (Mylyk and Threlkeld, 1974, Griffiths 1982). The formation of male and female reproductive organs is a complex process. Mutations in genes that control both mating and vegetative incompatibility have been shown to affect sex pheromone production, female sex organ development, fertilization and development of perithecia in several Ascomycetes, including *Neurosporacrassa, Podosporaanserina,* and *Cochliobolusheterostrophus,* as well as in the yeast *Saccharomyces cerevisiae* (Coppin et al. 1997).Why some *C. parasitica* mating pairs fail to produce perithecia when one isolate is the "female" (e.g. Schomberg x Bockenhauer), while the reverse mating combination (Bockenhauer x Schomberg) is successful, may be explained by the role of the *pro1* gene. According to Sun et al. 2009, the functional role of *pro1* in *C. parasitica* is distinctive among characterized $Zn(II)_2Cys_6$ binuclear cluster proteins in that it is required for both sexual development and asexual sporulation. Targeted disruption of this gene confirmed that it is required for female fertility but not for virulence. Mutations involving the proteins encoded for by *pro1,* and transcription factors produced by the gene *cpst12* may be involved in female infertility of the Schomberg isolate. Additional mating experiments, using the procedure developed here, along with molecular data, may lead to a better future understanding of sexual reproduction in populations of *C. parasitica*.

References

Anagnostakis, S.L. (1979). Sexual reproduction of Endothiaparasitica in the laboratory.Mycologia 71, 213-215.

Bramble, W.C. (1936).Reaction of chestnut bark to invasion by Endothiaparasitica. American Journal of Botany, 23, 89-94.

Burnett, J.H. (1968). Fundamentals of Mycology. New York: St. Martin Press. 546 p.

Coppin, E., Debuchy, R., Arnaise, S. & Picard, M. (1997). Mating types and sexual development in filamentous Ascomycestes.Microbiology and Molecular Biology Review, 61, 411-428.

Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. (9the ed.). (1974). Detroit Michigan: Difco Laboratories. 350 p.

Double, M.L. &Marshall,M.R. (2014). Strawberry amendment to potato dextrose agar to increase conidiation in Cryphonectriaparasitica.Acta Horticulture, 101, 81-84.

Double, M.L., MacDonald, W.L. & Taylor, G.M. (2014). Evaluation of Cryphonectriaparasitica isolates collected from the Great Smoky Mountains National Park. Acta Horticulture, 1019, 85-89.

Griffiths, A.J.F. (1982). Null mutants of the A and a mating type alleles of Neurosporacrassa. Canadian Journal of Genetics and Cytology, 24,167-176.

Hindal, D.F. (1982). Cultural considerations in Endothiaparasitica:State of the art. In: Smith, C.H. &W.L.MacDonald (Eds). Proceedings of the USDA Forest Service American Chestnut Cooperators Meeting. (pp. 200-209). Morgantown, WV: West Virginia University Books.

Jakobi, S.R. (2005). The effects of hypovirulentCryphonectriaparasitica inoculum, developmental stages of cankers, and time of year of wounding on the survival of the American chestnut.(Ph.D. dissertation). Morgantown, West Virginia: West Virginia University Press. 169 pp.

Kenaley, S.C., Double, M.L. & MacDonald, W.L .(2014). Effect of spore concentration on the establishment of cytoplasmic hypovirulent (HV), transgenic HV, and virulent isolates of Cryphonectriaparasitica.Acta Horticulture, 1019, 165-171.

Kuhlman, E.G. (1978).The devastation of American chestnut by blight. In: MacDonald, W.L., F. C. Cech, J. Luchok, & C. Smith , (Eds). Proceedings of the American Chestnut Symposium. (pp. 1-3).Morgantown, WV: West Virginia Univ. Books.

Kuhlman, E.G. (1982). Vegetative compatibility and hypovirulence in Endothiaparasitica: State of the art. In: Smith, H.C. &W. L. MacDonald (Eds). Proceedings of the USDA Forest Service American Chestnut Cooperators Meeting. (pp. 210-217). Morgantown, WV: West Virginia Univ. Books.

Kuhlman, E.G. & Bhattacharyya, H. (1984). Vegetative compatibility and hypovirulence conversion among naturally occurring isolates of Cryphonectriaparasitica. Phytopathology, 74, 676-682.

Lily V.G. & Barnett, H.L .(1951). Physiology of the Fungi. New York: McGraw-Hill.

MacDonald, W.L. & Double, M.L. (1978). Frequency of vegetative compatibility types of Endothiaparasitica in two areas of West Virginia. In: MacDonald, W.L. F.C. Cech, J. Luchok& C. Smith (Eds). Proceedings of the American ChestnutSymposium. (pp. 103-105). Morgantown, WV: West Virginia Univ. Books

Marra, R.E. & Milgroom, M.G.. (2001). The mating system of the fungus Cryphonectriaparasitica:selfing and self-incompatibility. Heredity, 86, 134-143.

Marshall, M.R. & Double, M.L. (2014).Strawberry amendment in potato dextrose agar to increase conidiation in Cryphonectriaparasitica.Acta Horticulture, 1019, 81-84.

McGuire, I.C., Marra, R.E .& Milgroom, M.G. (2004).Mating-type heterokaryosis and selfing in Cryphonectriaparasitica.Fungal Genetics and Biology, 41,521-533.

Milgroom, M.G. &Cortesi, P . (1999). Analysis of population structure of the chestnut blight fungus based on vegetative incompatibility types. Proceedings of the National Academy of Sciences, 96,10518-10523.

Milgroom, M.G., Wang K., Zhou, Y. & Kaneko, S. (1996). Intercontinental population structure of the chestnut blight fungus, Cryphonectriaparasitica.Mycologia, 88, 179-190.

Mylyk, O.M. &Threlkeld.S.F.(1974). A genetic study of female sterility in Neurosporacrassa. Genetics Research, 24, 91-102.

Rankin, W.H. (1914). Field studies on the Endothia canker of chestnut in New York State. Phytopathology, 4,233-259.

Sun, Q.,Choi, G.H. & Nuss, D. L. (2009). Hypovirus-responsive transcription factor gene pro1 of the chestnut blight fungus Cryphonectriaparasitica is required for female fertility, asexual spore development and stable maintenance of hypovirus infection. Eukaryotic Cell, 8, 262-270.

Tuite, J. (1969). Plant Pathological Methods. Fungi and Bacteria. Minneapolis, Minnesota: The Burgess Publ. Co. 239 pp.

USDA.(2014). SR 27 USDA national nutritional database for standard reference release 27. [online]. Available: http//:ndb.nal.usda.gov/ndb

Willey, R.L. (1980). Pathogenicity and sporulation of selected hypovirulent and virulent strains of Endothiaparasitica.[M.S. thesis].(111 pp.). Morgantown, West Virginia: West Virginia University Press.

Table I: CryphonectriaParasitica Isolates, their Geographic Origins and Mating Types Used in the Experiment

Isolate designation	Origin	Mating type
EP146	West Virginia	1
Schomberg	Wisconsin	1
7-5-1C	West Virginia	1
5-6-1F	West Virginia	1
6-3-1C	West Virginia	1
EP155	Connecticut	2
Bockenhauer	Wisconsin	2
6-7-1F	West Virginia	2
MN-1	Minnesota	2
MN-3	Minnesota	2

Table II: Nutrient Media Evaluated for Inducement of Perithecia and Ascospore Production by Sexually Compatible Isolates of CryphonectriaParasitica

Potato dextrose agar (PDA) (Difco Manual, 1953)
PDA amended with chestnut bark extract, pH6
Corn meal agar (Tuite, 1969)
Filter paper yeast extract (Tuite, 1969)
Asthana-Hawker (AH) medium (Tuite, 1969)
AH amended with 10-2 percent thiamin and biotin
Vogel medium (Tuite, 1969)
Sordaria mating agar (Carolina Biological Supply Company)
PDA amended with strawberry fruit extract (Double and Marshall, 2014)
PDA amended with 1 percent hemp oil
PDA amended with 10 percent hemp oil
Dialysis membrane on top of PDA (Jakobi, 2005)
Leonian agar (Tuite, 1969)
Leonian agar amended with 10-2 percent thiamin and biotin
Leonian agar amended with red oak extract
Leonian agar amended with 1 percent hemp oil
Leonian agar amended with 1 percent concentrated coconut milk
Leonian agar amended with 1 percent "light" (60 percent diluted) coconut milk

Table III: Cryphonectriaparasitica (Mating type 1 and 2) isolate combinations used to evaluate sexual reproduction on Leonian agar amended with 1 percent light coconut milk. The first isolate ("female") in the cross was spermatized with conidia from the "male" isolate. In most cases the crosses were then reversed. The number of successful crosses per isolate pairs, the length of time (d) required for the first appearance of perithecial necks, and the average rate of perithecia production (0-3) for each cross are indicated.

Isolate pairing	number of plates with perithecia	earliest appearance of perithecia (d)	average sporulation rating (0-3 scale)
Schomberg (1) x Bockenhauer (2)	0/5		
Bockenhauer (2) X Schomberg (1)	3/5	16	1.5
Schomberg (1) x EP155 (2)	0/5		
EP155 (2) xSchomberg (1)	7/7	13	3
EP146 (1) x Bockenhauer (2)	5/5	16	3
Bockenhauer (2) x EP146 (1)	1/5	35	1
EP146 (1) x EP155 (2)	5/5	14	2.5
EP155 (2) x EP146 (1)	5/5	20	3
6-3-1C (1) x 6-7-1 (2)	0/5		
6-7-1 (2) x 6-3-1C (1)	1/5	29	1
6-3-1C (1) x MN-1 (2)	2/5	30	1
7-5-1C (1) x 6-7-1 (2)	2/5	25	1
6-7-1 (2) x 7-5-1C (1)	2/5	47	1
6-7-1 (2) x Schomberg (1)	3/5	35	2
7-5-1C (1) x EP155 (2)	3/5	25	1
EP155 (2) x 7-5-1C (1)	4/4	18	1
EP146 (1) x MN-1 (2)	3/5	22	1.5
MN-1 (2) x EP146 (1)	2/5	41	1.5
MN-1 (2) x Schomberg (1)	4/5	18	1
MN-1 (2) x 7-5-1C (1)	4/5	30	1.5
7-5-1C (1) x MN-1 (2)	1/4	22	1
7-5-1C (1) x MN-3 (2)	3/5	23	1
MN-3 (2) x 7-5-1C (1)	0/5		
EP155 (2) x 6-3-1C (1)	4/4	18	2.5
6-3-1C (1) x EP155 (2)	3/5	36	1
EP155 (2) x 5-6-1F (1)	5/5	18	2.5
EP146 (1) x 6-7-1 (2)	2/4	36	1
6-7-1 (2) x EP146 (1)	4/5	30	1
EP146 (1) x MN-3 (2)	3/5	30	1